

THE ROLE OF PROTEIN CROSS-LINKING IN SOY FOOD TEXTURE

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ABSTRACT

Cross-linking in soy proteins is hypothesised to have an impact on the texture of tofu. *In vitro* incubation showed soy proteins and its two fractions, glycinin and β -conglycinin, were cross-linked using glutaraldehyde, formaldehyde, glyceraldehyde and transglutaminase (TGA). Increasing concentration of these carbonyl compounds and TGA, and temperature of the carbonyl compounds treatment, increased the reactivity of cross-linking. Glutaraldehyde was the most reactive in forming aggregated proteins, followed by formaldehyde and glyceraldehyde. Both carbonyl moieties of glutaraldehyde are believed to be essential for the rapid cross-linking reaction. In the unfractionated soy proteins, β -conglycinin had a higher reactivity than glycinin. In *in vitro* incubation using TGA, soy proteins served as good substrates for TGA, in which β -conglycinin was more susceptible to TGA than glycinin in the unfractionated soy proteins. The addition of TGA, and 1 and 2 mM glutaraldehyde prior to soymilk boiling *in situ* resulted in a small number of cross-linked proteins, which correspond to an increase in fracture force. The addition of glutaraldehyde after soymilk boiling resulted in a slight decrease in fracture force compared to the control. At higher concentrations of glutaraldehyde (15 and 30 mM), soy proteins were mostly cross-linked, regardless of addition before or after soymilk boiling. Highly cross-linked proteins resulted in a significant decrease in the fracture force. For TGA treatment, the fracture force was increased with increasing TGA concentration from 1000 to 5000 ppm, added either before or after soymilk boiling. However, the TGA treatment showed only a small quantity of cross-linking. It is hypothesised that TGA hydrolysed glutamine of proteins to glutamate and changed the functional properties of proteins. Upon examination of the microstructure, it was found that the TGA treatment resulted in a fine-stranded network, compact structure and less porosity. These characteristics resulted in a higher fracture force. In contrast, in the glutaraldehyde treatment, the network consisted of a higher porosity, loose network and diffuse structure, which gave lower fracture force. Thus, it appears that substrate modification to the structure of the soy proteins may have a greater impact than the number of cross-links. These findings are likely to have implications for production of soy products with a wide range of textures by manipulating the soy protein properties.

ABBREVIATIONS

A	acidic subunits
AU	arbitrary unit
B	basic subunits
BSA	bovine serum albumin
cm	centimetre
°C	degree Celcius
DTT	dithiothrietol
FDA	Food and Drug Administration
GPIO	general purpose interface bus
GDL	glucono- δ -lactone
Gln	glutamine
g	gram
hr	hour
kDa	kilodaltons
kV	kilovolt
Leu	leucine
L	litre
2-ME	2-mercaptoethanol
μ g	microgram
μ L	microlitre
MS	Microsoft
mA	milliampere
mg	milligram
mL	millilitre
mm	millimetre
mM	millimolar
min	minute
M	molar
MW	molecular weight
nm	nanometre
NCBI	National Center for Biotechnology Information

ln	natural logarithm
N	Newton
ppm	part per million
Phe	phenylalanine
pV	picovolt
RP-HPLC	reverse-phase high-performance liquid chromatography
rpm	revolutions per minute
SEM	scanning electron microscopy
SE-HPLC	size-exclusion high-performance liquid chromatography
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S	Svedburg unit
TPA	texture profile analysis
TGA	transglutaminase
TFA	trifluoroacetic acid
UTM	universal testing machine
V	volt

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CHAPTER 1

INTRODUCTION

1.1 Overview

Protein cross-linking is a term used to refer to the formation of protein-protein linkages between polypeptides of different proteins (inter-molecular cross-links) or within single polypeptides in a protein (intra-molecular cross-links) (Feeney and Whitaker 1988, Traut et al. 1989). The protein can be treated by various means, such as by heating or employing a chemical reagent or enzyme to introduce covalent bonds. This process not only changes the structure and functional properties of the proteins, but, more importantly, can change the texture of food (Gerrard 2002a).

For this study, tofu, or soybean curd, was selected as a model food protein system and subjected to chemical and enzymatic cross-linking. Tofu was selected because it is prepared by changing the nature of soy proteins to form a gel. Soy proteins in their native state do not form a gel; they must be heat-denatured and then coagulated to form the tofu (Liu 1997). By adding the cross-linking reagents (chemical or enzyme) before and after proteins are denatured, it was anticipated that the textural properties of the tofu would change to various degrees. Thus, the relationship between the degree of cross-linking and tofu texture could be established.

Before the cross-linking reagents were introduced into the tofu system, the effects of these reagents at a molecular level were examined. For this purpose, soy protein fractions were extracted and then incubated *in vitro* with various cross-linking reagents, in order to assess their efficiency. After assessing the efficiency of cross-linking reactions, the most reactive reagents were introduced into tofu *in situ* to find out how these cross-linking reactions in soymilk impacted tofu texture and whether this correlated with cross-linking patterns.

The term 'texture' has a broad meaning and its measurement relies on empirical tests. The textural properties of tofu were measured mechanically by adapting a method of (Bourne 1978) using the Instron Universal Testing Machine (UTM). Electron microscopy was also employed to examine changes in texture at the microscopic level.

This chapter gives an overview on how these cross-linking reagents can cross-link soy proteins and how they were expected to affect the textural properties of tofu.

1.2 The importance of soy proteins

On a dry matter basis, the protein and oil content of soybean seed varies from 39.5% to 50.2% and 16.3% to 21.6% respectively. The other minor components include phospholipids, vitamins, minerals, trypsin inhibitors, phytates, oligosaccharides and isoflavones (Liu 1997, Liu et al. 1995). Among other commercial legumes, the protein content in soybeans was the found to be the highest (Yaklich 2001). The consumption of soy foods in the human diet has increased because of their beneficial effects on nutrition and health (Friedman and Brandon 2001). This is due to the presence of a near perfect balance of all the essential amino acids, making soy a valuable protein source. Recently, the US Food and Drug Administration (FDA) approved a health claim that soy protein reduces the risk of coronary heart disease (Stewart 2005).

From a product development point of view, soy protein has been used in a wide range of food applications, mainly due to its functional properties. In recent years, new food products utilising soy protein have emerged to improve food functionality and applications. For example, when soy protein is blended with meat, it offers a unique quality and good flavour to formulated meat products (Hoogenkamp 2001). When added to dairy milk combined with polysaccharides, the soy proteins provide a good system to develop texturised milk pudding (Nunes et al. 2003). Indeed, the soy proteins were able to be manipulated in terms of their solubility according to the conditions i.e. temperature or salt level. This transformation could produce a product with specific properties to achieve the desired organoleptic properties, which include replacing meat or dairy products. Products such as texturised vegetable protein, soy cheese, soy yogurt

and meat analogues are available as alternative foods. All these products are manufactured by manipulating soy proteins under various conditions to meet specific functional properties of foods. In order to achieve this, numerous studies are continually being undertaken with regard to the physicochemical properties of soy proteins (Stewart 2005).

1.3 Characterisation of soy proteins

Using ultracentrifugation, soy proteins have been classified into four fractions, namely 2S, 7S, 11S and 15S globulins, according to their sedimentation rate when dissolved in a pH 7.6, 0.5 M ionic strength buffer (Koshiyama 1969). The approximate fraction compositions and their components are presented in Table 1.1.

Table 1.1: Approximate compositions of ultracentrifuge fractions of water-extractable soybean proteins

Fraction	Fraction (%)	Components	Molecular weight (kDa)
2S	22	Trypsin inhibitors Cytochrome c	8-21 12
7S	37	Hemagglutinins Lipoxygenases β -Amylase β -Conglycinin	110 102 67.7 180-210
11S	31	Glycinin	350
15S	11	Polymerised glycinin (Nielsen 1985)	600

Adapted from Wolf and Cowan (1975).

Among the four fractions, the 11S and 15S globulins are the two major protein fractions. The 11S and the 15S globulins are also known as soybean glycinin and β -conglycinin (Nielson 1985). This classification is based on the sedimentation coefficients only. A more comprehensive study on the characterisation of glycinin and β -conglycinin has taken place at the molecular level using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the individual subunit mobilities (Mujoo et al. 2003, Nishinari et al. 1991, Petruccielli and Anon 1995).

Glycinin and β -conglycinin, which account for about 65–85% of total seed protein, are found to be the most important soy proteins and have been utilized and modified for various purposes, such as for food functionalities (Liu 1997).

1.3.1 Glycinin – 11S fraction

The molecular structure of glycinin is schematically represented in Figure 1.1. Glycinin is an oligomeric protein and has a hexameric structure with a molecular mass around 350 kDa. Each hexamer consists of two trimers, and the trimer consists of three monomers. In the hexamer, six monomers are arranged in a trigonal antiprism structure, as determined by x-ray scattering (Plietz et al. 1983). Glycinin consists of acidic (A) and basic (B) subunits, which have an acidic and a basic isoelectric point respectively.

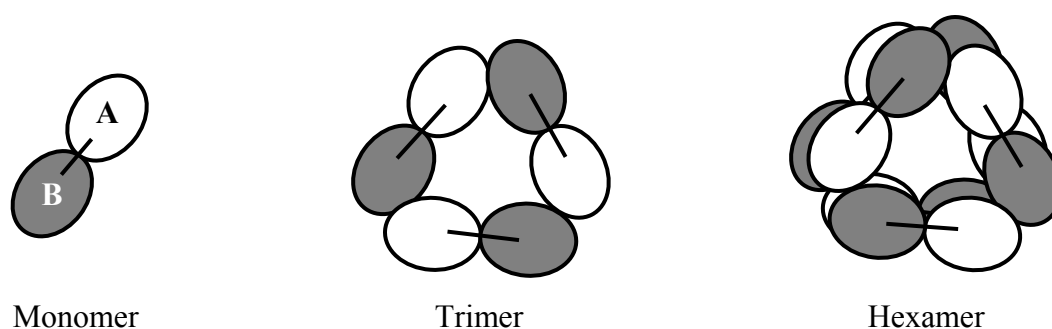


Figure 1.1: Schematic molecular structure of glycinin represented by monomer, trimer and hexamer. A and B are the acidic and basic polypeptides. The bar connecting A and B is a disulfide bond. Adapted from Renkema et al. (2001).

In an earlier study, Kitamura et al. (1976) reported four different types of acidic (A_1 - A_4) and basic polypeptides (B_1 - B_4) in soy proteins. However, in later studies, the monomeric components that constitute the A subunits were found to consist of six acidic polypeptide chains (37-45 kDa; $pI = 4.2$ - 4.8), whilst the B subunit was made-up of six basic polypeptide chains (18-20 kDa; $pI = 8.0$ - 8.5) (Peng et al. 1984, Utsumi et al. 1981, Wolf and Briggs 1985). The acidic and basic polypeptide chains are linked with a disulfide bond. Only acidic polypeptide A_4 was not linked by a disulfide bond to other basic polypeptides (Staswick et al. 1984). Their quaternary structure is stabilized by electrostatic and hydrophobic interactions, and also by disulfide bonds (Badley et al. 1975, Peng et al. 1984).

The A and B subunits, and their polypeptide chains can undergo dissociation with urea, plus a reducing agent such as 2-mercaptoethanol. The schematic mechanism of dissociation of glycinin ($A-S-S-B$)₆ into subunits and then into polypeptides is shown in Figure 1.2. The acidic (A) and basic (B) polypeptides have a molecular weight of 34-44 kDa and 20 kDa respectively (Yaklich 2001).

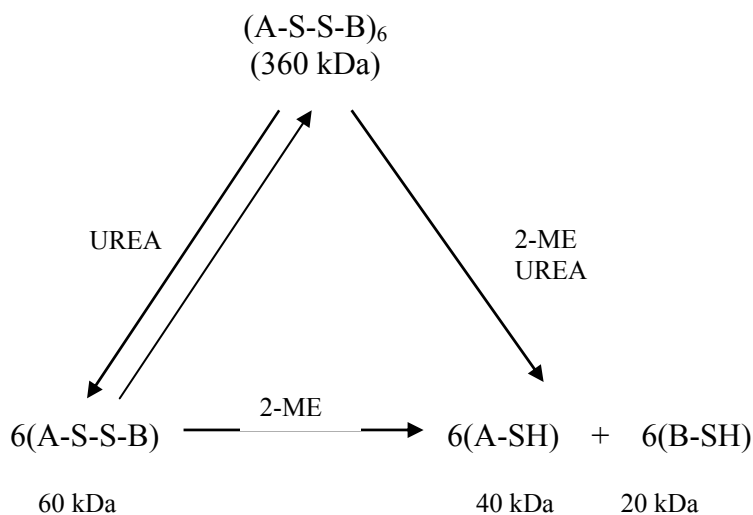


Figure 1.2: Schematic mechanism of glycinin dissociation into subunits and polypeptides by urea and 2-mercaptoethanol. 2-ME = 2-mercaptoethanol; A = acidic polypeptides; B = basic polypeptides; S-S = disulfide bond. Adapted from Nielson (1985).

Other than by using reducing agents such as 2-mercaptoethanol, the dissociation of glycinin can be induced by other factors, such as changing the ionic strength, pH or temperature (Peng et al. 1984). For instance, with ultracentrifugal analysis, upon lowering the ionic strength from 0.5 to 0.03 M in acidic condition (pH 3.8), the glycinin (11S globulin) starts to appear in the 7S globulin fraction (Lakemond et al. 2000). At 0.03 M ionic strength, in slightly alkaline conditions (pH 8.6), the glycinin (11S globulin) starts to dissociate into smaller units that appear in the 7S and 2S fractions (Eldridge and Wolf 1967, Koshiyama 1972, Lakemond et al. 2000).

1.3.2 β -Conglycinin – 7S fraction

The β -conglycinin fraction is comprised of three major subunits, namely α' , α , and β , and a minor subunit γ (Thanh and Shibasaki 1977). The molecular structure of β -conglycinin mainly consists of α' , α , and β subunits arranged as schematically presented in Figure 1.3. β -Conglycinin is a trimeric glycoprotein with a molecular mass of 180-210 kDa. Subunits α' , α , and β have been reported to have molecular masses of 57-72, 57-68, and 45-52 kDa respectively (Yamauchi et al. 1981). The subunits are associated via hydrophobic interactions and hydrogen bonding without any disulfide bonds (Thanh and Shibasaki 1978). When the ionic strength is more than 0.5 M, the trimeric structure of β -conglycinin is completely stabilized. The isoelectric point of β -conglycinin is 4.64 (Koshiyama 1983). For the subunits, the isoelectric point of the β subunit, consisting of four components (β_1 - β_4) is about 5.8 – 6.2, whereas the α and α' subunit consists of single components, having isoelectric points of 5.2 and 5.3, respectively (Thanh and Shibasaki 1976).

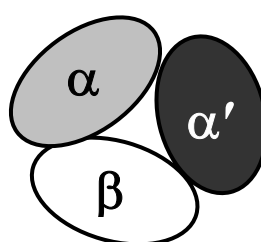


Figure 1.3: Trimeric molecular structure of $\alpha\alpha'\beta$ type β -conglycinin. Adapted from Renkema (2001).

Since β -conglycinin is heterogeneous, seven quaternary structures have been isolated and identified in various combinations, namely $\alpha'\beta\beta$; $\alpha\beta\beta$, $\alpha\alpha'\beta$, $\alpha\alpha\beta$, $\alpha\alpha\alpha$, $\alpha\alpha\alpha'$ (Thanh and Shibasaki 1976) and $\beta\beta\beta$ (Sykes and Gayler 1981, Yamauchi et al. 1981). β -Conglycinin is also able to associate and dissociate in response to changes in ionic strength and pH. With ultracentrifugal analysis at neutral pH and an ionic strength of > 0.5 M, β -conglycinin fractionates with the 7S globulin. When the ionic strength is 0.01 M at pH 12.0, the β -conglycinin (7S globulin) dissociates and appears in the 2S globulin fraction (Thanh and Shibasaki 1979). Under free-salt conditions at lower ionic strength (pH 7.6, $I = 0.01$ M), α and α' subunits dissociate, but β subunit remained stable (Iwabuchi et al. 1991).

Heating at various ionic strengths affects the association and dissociation behaviour of β -conglycinin. Heating at an ionic strength of 0.5 M led to β -conglycinin forming aggregates. However, β -conglycinin dissociates into subunits when heated at a near zero ionic strength (Iwabuchi and Yamauchi 1984).

1.4 Functional properties of soy proteins

In food biochemistry, the functional properties of proteins are often described as: “the influence of the physicochemical properties of proteins on the behaviour of the food system during preparation, processing, storage, and consumption that leads to its quality and sensory attributes” (Zayas 1997). The physicochemical properties are controlled by composition, structure and conformation of proteins (Kinsella 1979). The functional properties of soy proteins and their modes of action are summarised in Table 1.2. Among the functional properties possessed by the soy proteins is gelation, which is thought to govern the textural properties of tofu.

Table 1.2: Functional properties of soy proteins in food systems.

Functional property	Mode of action	Food system used	Products
Solubility	Protein solvation, pH dependent	Beverages	F, C, I, H
Water absorption and binding	Hydrogen bonding of water, entrapment of water, no drip	Meats, sausages, breads, cakes	F, C
Viscosity	Thickening, water binding	Soups, gravies	F, C, I
Gelation	Protein matrix formation and setting	Meats, curds, cheese	C, I
Cohesion-adhesion	Protein acts as adhesive material	Meats, sausages, baked goods	F, C, I
Elasticity	Disulfide links in gels deformable	Meats, bakery	I
Emulsification	Formation and stabilization of fat emulsion	Sausages, bologna, soup, cakes	F, C, I
Fat adsorption	Binding free fat	Meats, sausages, doughnut	F, C, I
Flavour-binding	Adsorption, entrapment, release	Simulated meats, bakery	C, I, H
Foaming	Forms stable films to entrap gas	Whipped toppings, chiffon deserts	I, W, H
Colour control	Bleaching by lipoxygenase	Breads	F

F = soy flour; C = soy protein concentrate; I = soy protein isolate; H = soy protein hydrolysate; W = soy whey. Adapted from Kinsella (1979).

1.5 Gelation mechanism of soy proteins

In general, gelation of proteins is an important step in giving food products a desirable texture. Thermal denaturation followed by aggregation and gelation are the important steps to ensure that all proteins in the suspension are incorporated into the gel network (Aguilera 1995, Clark and Ross-Murphy 1987).

In tofu manufacturing, the gelation of soy proteins is induced by heat denaturation of soy proteins followed by aggregation and cold-setting gelation to form a tofu gel (Kohyama et al. 1995). During cold-setting gelation, a three-dimensional network of protein aggregates is formed that may hold water, lipids, sugars, flavours and other ingredients (Kinsella 1979). The degree of random aggregation and entanglements determines the type of gel structure formed, which can be characterised by viscosity, plasticity and elasticity (Hermansson 1986, Kinsella 1979).

The principle behind the heat-denaturation is to unfold the proteins. Instead of having a compact structure, the protein structures become more diffuse, and the interiors of the protein molecules are exposed. For example, functional groups such as sulfhydryl, hydrophobic residues, carbonyl groups, amide groups of peptide bonds and side-chain amide groups become exposed, which can affect the network structure (Wang and Damodaran 1991). The association-dissociation and aggregation during heat-denaturation also leads the protein molecules to form strands. With the interaction of protein molecules, strands and aggregates, a three-dimensional network of protein is formed (Hermansson 1986).

The mechanism of soy protein gelation is determined mainly by the heat-induced association-dissociation behaviour of soy proteins. The gelling mechanism was found to be different for glycinin and β -conglycinin fractions. The gelation mechanism in tofu was affected by the presence of both glycinin and β -conglycinin fractions. For instance, the compositional differences in terms of the ratio of glycinin to β -conglycinin (Kwanyuen et al. 1998), variations in subunit concentrations within fractions (Nakamura et al. 1984a), and differences in amino acid profiles (Serretti et al. 1994), were able to change the quality of the tofu. The ratio of glycinin to β -conglycinin was reported to vary with soybean variety and also depended on soybean maturity (Cai and Chang 1999, Ji et al. 1999, Min et al. 2005, Mujoo et al. 2003, Tanteeratarm et al. 1989). A higher ratio of glycinin to β -conglycinin contributed to an increase in hardness of the tofu gel (Kang et al. 1991).

1.6 Microstructure and texture of tofu

The textural properties of tofu play an important role in determining its acceptance by the consumer. Tofu can be classified into three categories namely, i) soft (silken), ii) firm, and iii) extra firm tofu, as determined by the water content. They are prepared by the same procedure, except for the differences in the water to soybean ratio, the type and concentration of coagulant and the amount of whey pressed out (Liu 1997).

In heat-induced gelation, the protein suspension changes from a stable colloidal state to a three-dimensional network following the order: denaturation, aggregation, gelation (Aguilera 1995, Clark and Ross-Murphy 1987, Schmidt 1981). In tofu manufacturing, the gelation mechanism involves two main steps: i) protein denaturation as a result of heat; and ii) hydrophobic aggregation as a result of coagulation. In the first step, the hydrophobic regions of the soy proteins in the native state, which are buried inside the molecule's structure, are exposed. Since the denatured proteins are negatively charged (Kohyama and Nishinari 1993), the presence of positive charges from coagulant, i.e Ca^{2+} ions, neutralise the net charges of the proteins. In the second step, these neutralised proteins are aggregated predominantly by hydrophobic interactions. The hydrophobic interactions are thought to play a role in the gel structure (Kohyama et al. 1995), and the formation of a gel was found to occur by random aggregation (deMan et al. 1986). A model of the gelation mechanism is schematically presented in Figure 1.11.

In this soy gel system, the soy proteins can be manipulated to change the gel texture. One such modification is the addition of polysaccharides into the soy protein system. Polysaccharides, such as carrageenan, are reported to be capable of changing the functional properties of soy proteins, leading to changes in textural properties. When carrageenan was added to the soy protein isolate for gel formation, the gel had a higher gelation kinetic constant and higher viscoelasticity than the non-treated gel (Ortiz et al. 2004). The effect on tofu was also investigated, and adding carrageenan during tofu manufacturing resulted in a significant increase in tofu hardness (Karim et al. 1999). Apart from carrageenan, chitosan was also added in tofu preparation, and with the resulting tofu having higher gel strength and storage stability (Chang et al. 2003).

Under microscopic observation of a heat-induced gel, the network structure of protein gels exhibits a certain degree of order, essentially divided into two types: i) a fine stranded network, and ii) a coarse-aggregated network (Hermansson 1994). In the fine-stranded network, the proteins are attached to each other like “a string of beads”, and form an ordered gel (Nakamura et al. 1984b). This type of gel is usually transparent and the thickness of each strand is about 12-15 and 10-14 nm for glycinin and β -conglycinin respectively (Hermansson 1994, Hermansson 1986). Coarse networks are non-transparent and are thought to be formed by random aggregation of proteins into clusters, which aggregate to thick strands (Doi 1993). When the gel structure becomes coarser, the ability of gels to hold water decreases (Hermansson 1994, Hermansson 1986). The molecular structures of fine-stranded and the coarse-aggregated networks are schematically presented in Figure 1.4.

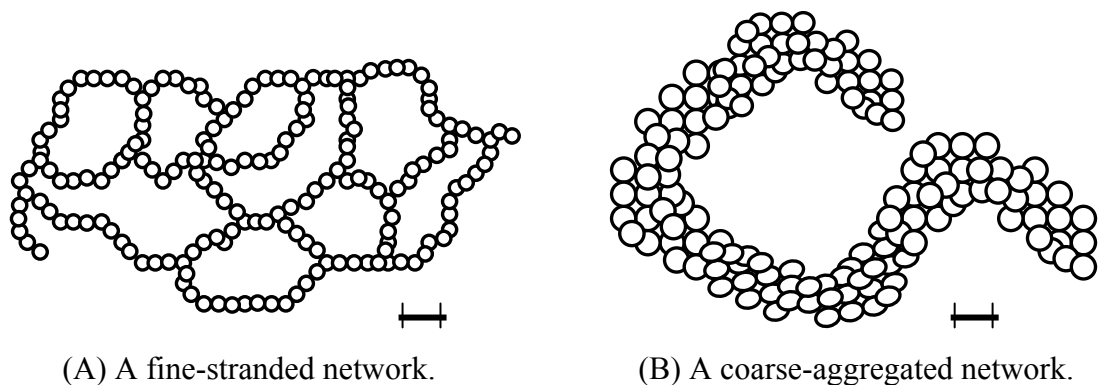


Figure 1.4: Schematic diagram of a fine-stranded network (A) and coarse-aggregated network (B). Scale bar indicates approximately 40-50 μm . Adapted from Renkema (2001).

Whether a gel forms from a fine strand network or a coarse-aggregated network depends on the gel formation conditions. The gel can become coarser if the pH approaches the isoelectric point of the proteins, or when the ionic strength is increased (Doi 1993). For example, a fine stranded network structure of glycinin was found between pH 7.0-7.6 (Hermansson 1985, Mori et al. 1986, Nakamura et al. 1984b).

In the case of globular proteins, changing the protein concentration, pH or ionic strength, caused the network structure to change and indirectly contributed to the gel strength, as illustrated in Figure 1.5.

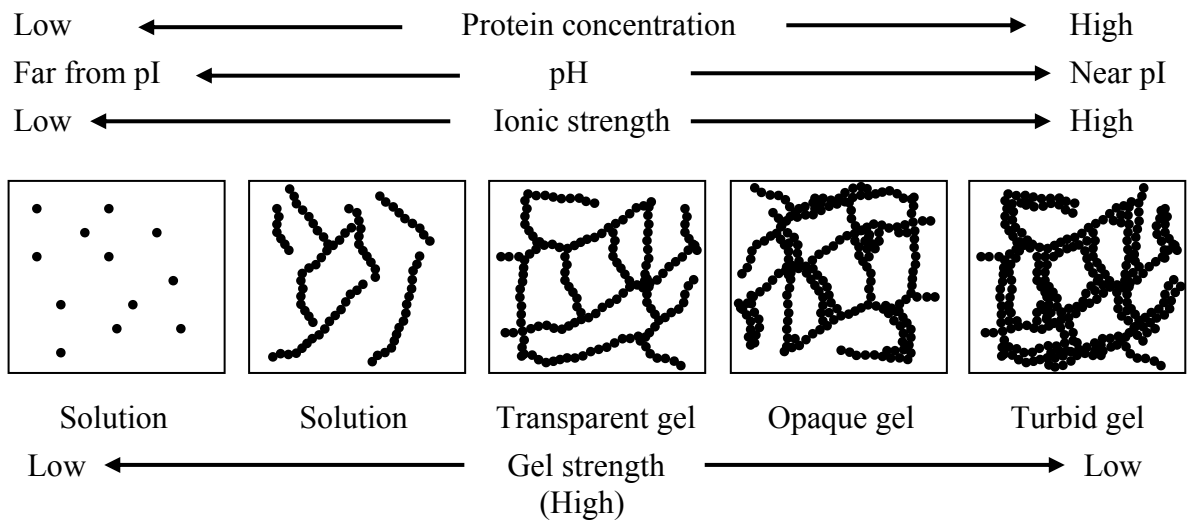


Figure 1.5: A typical model for the formation of protein network structure by changing the protein concentration, pH or ionic strength. Adapted from Hegg (1982) and Oakenfull et al. (1997).

Conditions that can change the microstructure and texture of tofu are summarised as follows:

- i) Soybean characteristics such as variety, growing location and storage conditions, affecting glycinin to β -conglycinin ratio (Bhardwaj et al. 1999, Cai and Chang 1999, Skurray et al. 1980, Tsai et al. 1981, Wang et al. 1983).
- ii) Soymilk processing conditions, such as soaking, water-to-bean ratio, grinding conditions and soymilk boiling conditions (Beddows and Wong 1987, Cai et al. 1997, Shih et al. 1997).
- iii) Coagulant characteristics, such as coagulant types and their concentrations (deMan et al. 1986, Kao et al. 2003, Sun and Breene 1991).

- iv) Coagulating variables, such as the method of mixing coagulant solution into soymilk, soymilk temperature and the amount of coagulant added into the soymilk (Evans et al. 1997, Hou et al. 1997).

1.7 Cross-linking reactions in food proteins

Proteins have functional groups that are often modified to change their properties in protein-based food; for example, texture, flavour, colour or solubility (Hoogenkamp 2001, Tropini et al. 2000). An important modification involving protein-protein interaction is protein cross-linking. The major pathways of protein cross-linking involving amino acid residues during food processing are schematically presented in Figure 1.6 (Gerrard 2002a).

Generally, the introduction of protein cross-linking depends on food processing conditions, such as heating, changing of pH or application of enzymes. Specific conditions result in specific types of cross-linking reaction in proteins. The most common types of cross-links found during food processing are disulfide bonds, cross-links derived from dehydroprotein, from tyrosine, from the Maillard reaction and by enzymatic reaction (Gerrard 2002a).

In this study, two types of cross-links were explored: i) Maillard-type cross-linking by addition of reactive carbonyl compounds, and ii) enzymatic cross-linking using the enzyme transglutaminase (TGA). They were chosen because they were able to modify the food proteins to produce desirable changes in textural properties of foods via two different mechanisms (Gerrard et al. 2001, Hill and Easa 1998). In Maillard-type cross-linking, the reaction between food proteins and carbonyl compounds can achieve desirable organoleptic properties, including texture (Mohammed et al. 2000). TGA catalyses the cross-linking of food proteins and this has been shown to lead to changes in food texture (Schwenke 1997, Soares et al. 2004, Zhu et al. 1995).

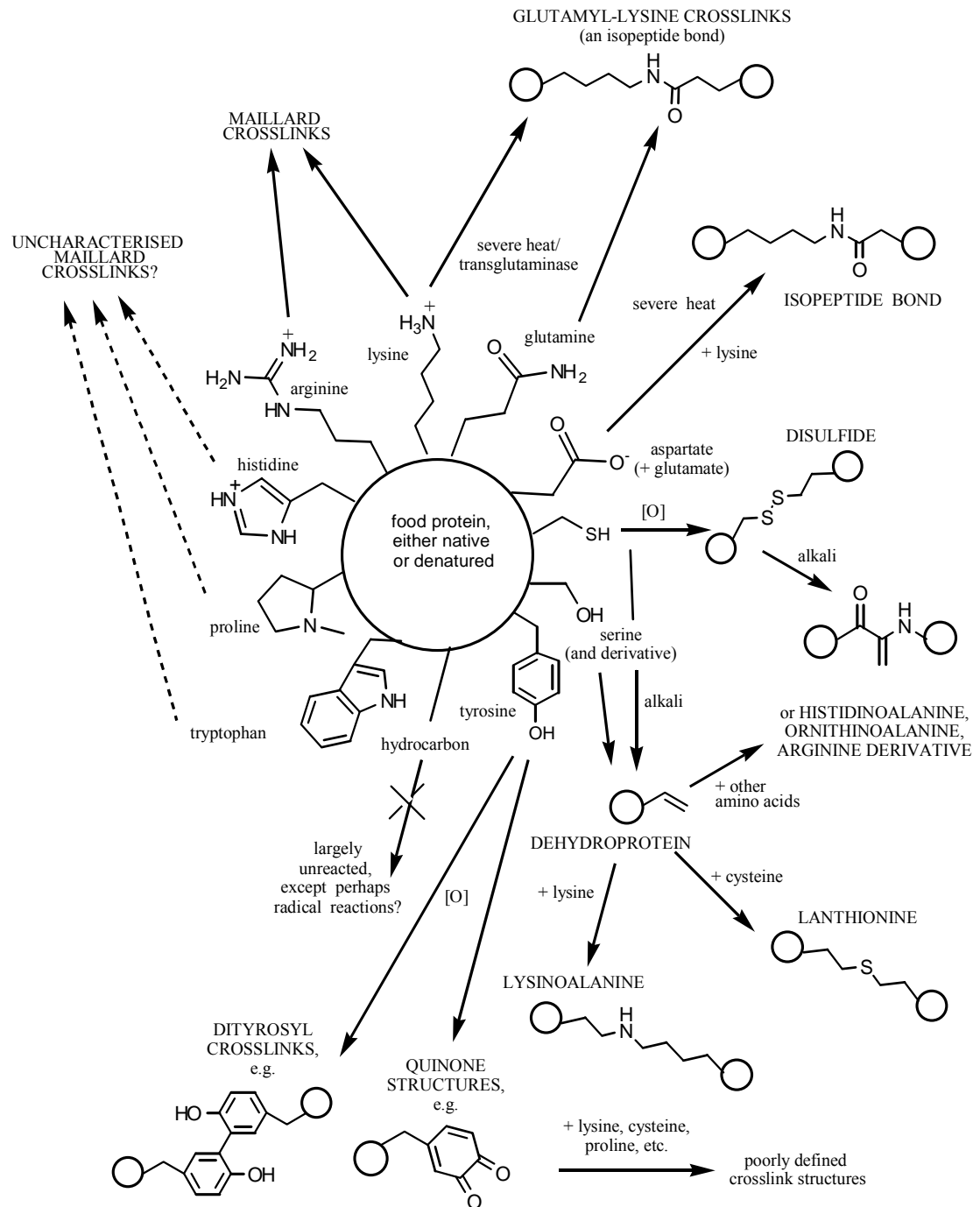


Figure 1.6: A schematic diagram of cross-linking reactions in food proteins during processing. Adapted from Gerrard (2002a).

1.7.1 Maillard-type cross-linking

The Maillard reaction has played an important role in food processing using heat treatment. Upon heating, the reaction results in the formation of aroma, taste and colour in foods, which could be beneficial or detrimental to food quality (Martins et al. 2001).

Maillard-type cross-linking may occur between amino acid residues in proteins and carbonyl groups, often derived from food sugars. Carbonyl compounds such as formaldehyde, glutaraldehyde, glyoxal and glyceraldehyde can promote inter- and intra-molecular cross-linking in proteins (Acharya et al. 1988, Gerrard et al. 2002b, Marquie 2001). In particular, the ϵ -amino group of lysine was found to be the primary reactive site between proteins and aldehyde. As a result, multimeric proteins were formed by covalent aggregation (Gerrard et al. 2003a, Gerrard et al. 2003b, Nagaraj et al. 1996). Since soy proteins have a higher lysine content (about 70 mg/g protein) than other cereal proteins (Liu 1997, Zarkadas et al. 1997), it was predicted that lysine residues would be more easy to cross-link than cereal proteins via the Maillard reaction.

1.7.2 Enzymatic cross-linking using TGA

TGA catalyses covalent cross-linking, which leads to polymerisation of food proteins by forming ϵ -(γ -glutamyl)lysyl bonds (Motoki and Seguro 1998). The enzyme catalyses an acyl-transfer reaction between the γ -carboxyamide group of a peptide-bound glutamine residues and the ϵ -amino group of lysyl residues of protein or peptide chains (Folk and Finlayson 1977). TGA can also catalyse incorporation of various primary amines into proteins (Folk 1983). Further details on TGA activity are discussed in section 1.8.2.

1.8 **Choice of cross-linking molecules**

1.8.1 Chemical reagents

Formaldehyde, glyceraldehyde and glutaraldehyde were selected for their varying capacity to cross-link a number of proteins (Acharya et al. 1988, Gerrard et al. 2002b, Gerrard et al. 2003b, Marquie 2001). Comparing the reactivities among these reagents in Maillard-type cross-linking, glutaraldehyde has been found to cross-link protein most efficiently, followed by formaldehyde and glyceraldehyde (Gerrard et al. 2003b). Further details on the mechanism and implication of these molecules on the proteins are summarised in Figures 1.7, 1.8 and 1.9.

1.8.1.1 Formaldehyde

Despite being the simplest cross-linking reagent, formaldehyde has been shown to cross-link a number of the side chain amino acid residues of proteins, in particular, lysine, to form oligomers (Galembeck et al. 1977, Gerrard et al. 2002b). By introducing covalent cross-links with formaldehyde, the functional properties of food proteins are modified (Singh 1991). For example, a film produced by formaldehyde cross-linked gelatin showed an increase in tensile strength and had greater thermal stability, with an increase in the melting point, due to the high degree of covalent cross-linking (de Carvalho and Grosso 2004). The formaldehyde was shown to react with the side chains of lysine, cysteine and histidine groups of the proteins to establish new covalent bonds (Galiotta et al. 1998, Marquie et al. 1995).

A significant increase in the puncture strength of protein film was also observed when defatted cottonseed flour was treated with formaldehyde (Marquie et al. 1995). Formaldehyde cross-linking of wheat gluten and soy protein isolate caused an increase in tensile strength (Ghorpade et al. 1995, Micard et al. 2000, Rhim et al. 2000). The proposed mechanisms of formaldehyde cross-linking via lysine residues are presented in Figure 1.7.

It has been suggested that formaldehyde forms a methylene bond between two lysine residues in each cross-link (Marquie 2001). However in a separate study, the rate of cross-linking and the rate of lysine residue loss did not correlate, suggesting that another mechanism might be occurring or that more than one mechanism may be operating (Gerrard et al. 2002b).

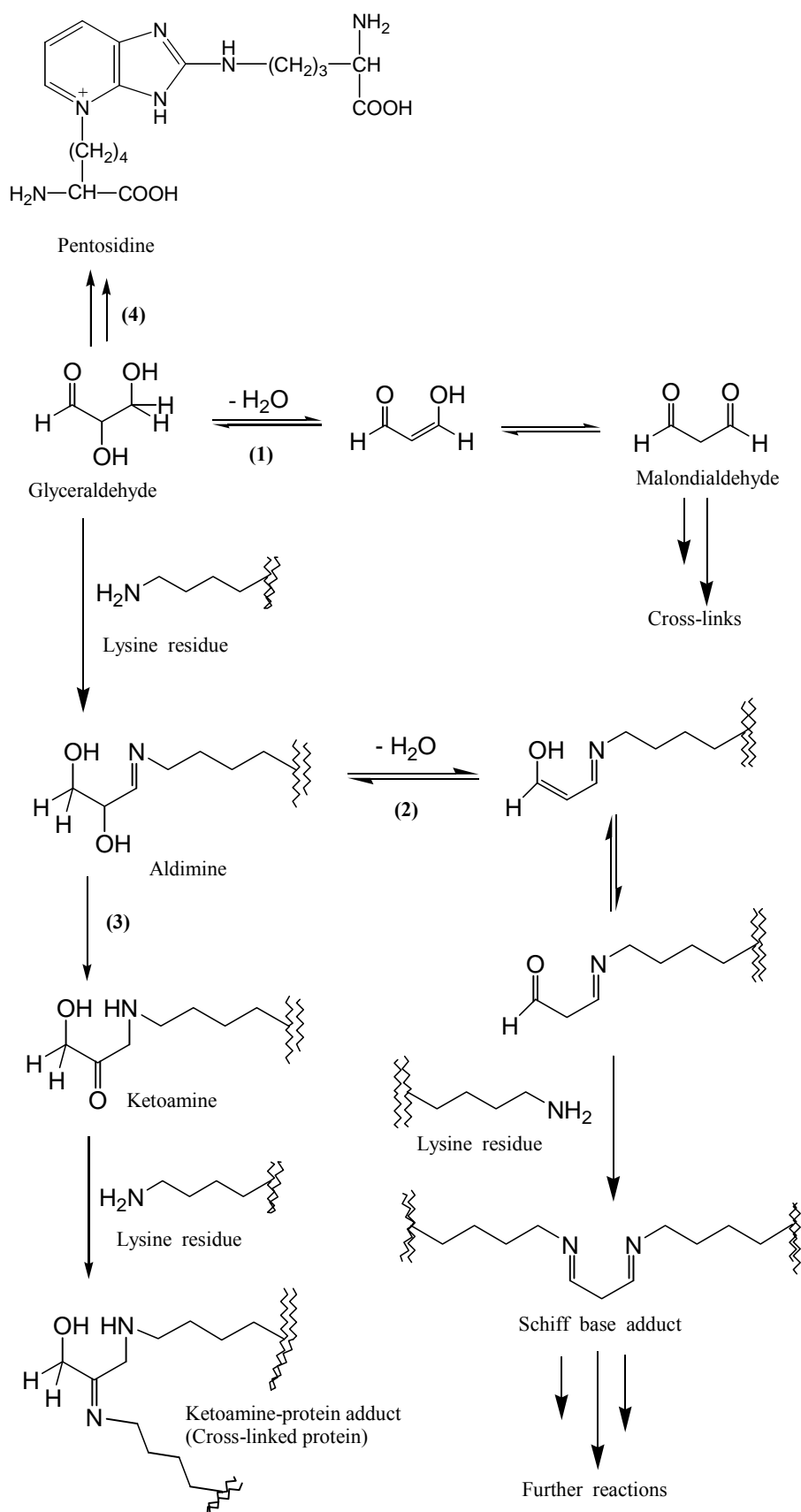


Figure 1.8: Proposed mechanisms for the cross-linking of proteins by glyceraldehyde via the Maillard reaction. Route 1 and 2 were adapted from Gerrard et al. (2002b); Route 3 was adapted from Acharya et al. (1988). Route 4 was adapted from Chellan and Nagaraj (2001) and Henle et al. (1997).

Glyceraldehyde by itself can undergo dehydration to generate malondialdehyde (Route 1) which may subsequently cross-link the proteins (Miyata et al. 1998). Another possible pathway is dehydration of N-substituted glycosylamine to form a Schiff base adduct (Route 2) (Gerrard et al. 2002b). Another aspect of the Maillard reaction of protein is rearrangement of N-substituted glycosylamine to generate keto-amine adducts (Amadori products) as indicated in Route 3 (Acharya et al. 1988). If the carbonyl function of the keto-amine exhibits reactivity towards amino groups of proteins, this may provide one of the possible pathways for the formation of cross-linked proteins (Acharya et al. 1988). When glyceraldehyde generate pentose, it links one arginine and one lysine residue together to form pentosidine (Route 4) (Chellan and Nagaraj 2001, Henle et al. 1997).

1.8.1.3 Glutaraldehyde

Glutaraldehyde is a symmetrical α -dicarbonyl compound and has two reactive moieties. It has been used extensively as a cross-linking reagent in various types of proteins for the production of biopolymers (Hernández-Muñoz et al. 2004, Kikuchi et al. 2004, Marquie et al. 1995, Ustunol and Mert 2004). Similarly, glutaraldehyde was shown to cross-link ribonuclease, bovine serum albumin (BSA) and casein to form multimeric compounds (Meade et al. 2003, Silva et al. 2004). When applied to wheat flour dough, glutaraldehyde cross-linked with the albumin and globulins of wheat proteins, which resulted in an increase in the relaxation times of the dough, as compared to controls, and had a significant impact on crumb strength and texture of bread (Brown 2002, Gerrard et al. 2002b, Gerrard et al. 2003a, Gerrard et al. 2003b). Proposed cross-linking mechanisms of glutaraldehyde with protein are schematically presented in Figure 1.9.

The most probable scenario is that glutaraldehyde reacts with lysine or hydroxylysine residues of proteins to form covalent cross-links (Silva et al. 2004). In alkaline conditions, glutaraldehyde is polymerised to form unsaturated polyglutaraldehyde that could subsequently react with reactive lysine. For example, when glutaraldehyde reacted with cottonseed protein, the cross-link molecules were thought to comprise eight glutaraldehyde molecules forming covalent bonds between two lysyl groups (Korn et al. 1972, Marquie 2001).

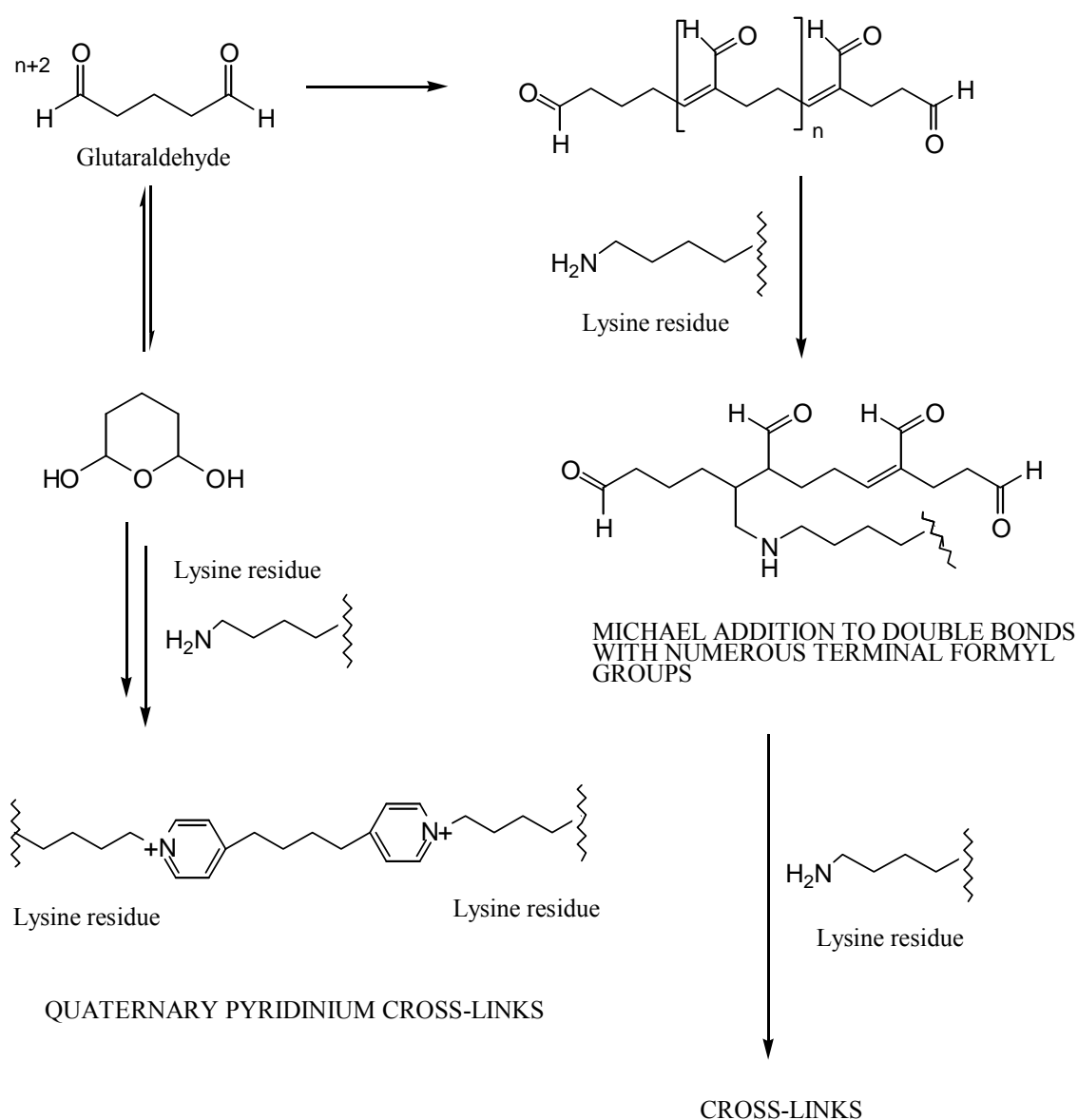


Figure 1.9: Proposed mechanisms for cross-linking of proteins by glutaraldehyde via the Maillard reaction. Adapted from Hermanson (1996).

The reactivity of glutaraldehyde was also found to be dependent on the specific structure of the proteins. For instance, when using BSA, a globular protein, the accessibility of glutaraldehyde to the lysine residues may be restricted, owing to the compactness of the protein molecules (Silva et al. 2004).

Besides having two reactive moieties, the rapid reaction could also be due to the specific chemical mechanism which occurs with glutaraldehyde cross-linking, which

has been proposed to result in heterocyclic pyridinium cross-links as presented in Figure 1.8 (Gerrard et al. 2002b, Meade et al. 2003).

1.8.2 Enzymatic reagent – Transglutaminase (TGA)

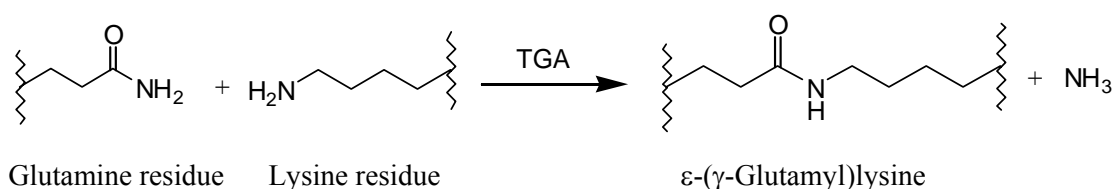
Microbial TGA (in this study called TGA) is a monomeric protein (Yokoyama et al. 2004). The primary structure consists of 331 amino acid residues, with a molecular weight of 37,842 Da and the active site includes a single cysteine residue, Cys64 (Kanaji et al. 1993, Washizu et al. 1994).

Over the years, a wide range of foods and food products have been treated with TGA, including fish products, meat products, dairy products, gelatin, baked goods, noodles and pasta (Kuraishi et al. 2001, Motoki and Seguro 1998, Zhu et al. 1995). The optimum conditions for using microbial transglutaminase are in the pH range from 4 to 9 and in the temperature range from 0 to 70°C (Motoki and Seguro 1998, Nielsen et al. 1995) which is suitable for most food processing. The benefits derived from TGA treatment include texture improvement, meat restructure, gel formation and incorporation of lysine into the product to increase the nutritional value (Folk 1983, Muguruma et al. 2003, Nio et al. 1985, Serrano et al. 2004). The polymerization is not limited to homologous proteins, but extends to heterologous protein systems, such as between milk, meat, soybean and wheat proteins (Babiker 2000, Basman et al. 2003, Kuraishi et al. 1997, Kuraishi et al. 2001, Larre et al. 2000, Motoki and Seguro 1998). The formation of ϵ -(γ -glutamyl)lysyl isopeptide bonds has been reported to strengthen the surimi gel (Kumazawa et al. 1995).

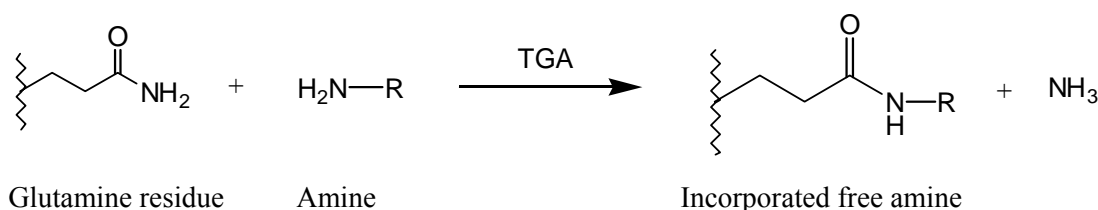
The efficiency of TGA activity is dependent on the macromolecular flexibility of the protein substrate. For example, the flexibility of casein protein allows it to serve as a good substrate for TGA, but in many globular proteins, TGA was found to be inefficient until the native proteins had been denatured. In contrast, despite the low lysine content in gelatin, it is a good substrate for TGA because of the flexibility associated with its random coil structure, as long as the collagen glutamine residues are not converted into glutamic acid (Babin and Dickinson 2001).

The catalytic activities of TGA in food protein are classified into three types of reaction: i) covalent cross-linking, ii) amine incorporation, and iii) deamidation. These are schematically presented in Figure 1.10.

i) Covalent cross-linking



ii) Amine incorporation



iii) Deamidation

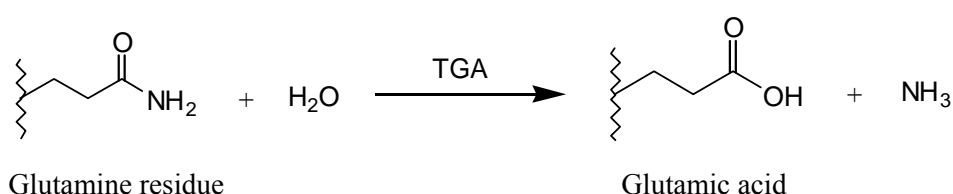


Figure 1.10: The proposed reactions catalysed by transglutaminase. TGA = transglutaminase. Adapted from Sharma et al. (2001). i) Covalent cross-linking: The formation of ϵ -(γ -glutamyl)lysine bonds in which glutamine and lysine residues are cross-linked. The ϵ -amino groups of lysine residues in proteins can act as the primary amines, yielding inter- and intra-molecular covalent cross-links (Motoki and Seguro 1998). ii) Amine incorporation: If only the glutamine residue is available for reaction, TGA catalyses the incorporation of primary amines into proteins (Nonaka et al. 1996). iii) Deamidation: In the absence of amine groups, water becomes an acyl acceptor. TGA catalyses the hydrolysis of the γ -carboxylamide group of the glutamyl residue, resulting in deamidation of the glutamine residue (Ando et al. 1989).

1.9 Textural properties - fracturability

As mentioned earlier (section 1.1), cross-linking in proteins has a profound effect on the texture of the food processed. How the texture is measured will depend on the food in question. With tofu, the texture measurement ideally relates to the movement of the jaw, where a force is applied in chewing the food materials from the increasing and decreasing pressure of individual teeth (Dobraszczyk and Vincent 1999). To translate the force of chewing and relate it to the texture, an empirical measurement has been attempted based on the rheology of food deformation.

As rheology is defined as “the study of the deformation and flow of a matter”. The material testing procedures consist of small deformation non-destructive tests and larger strain destructive tests (Sharma et al. 2000). The non-destructive test is very useful for characterising various network structures in many foods including cheese and tofu. The required force to deform the material is measured as a function of time, or displacement, in order to achieve the texture profile analysis (TPA) by a uniaxial compression test (Bourne 1978). The TPA results in a number of textural parameters. The fracturability at the maximum deformation was chosen which occurred at 15 mm, targeted distance.

1.10 Gelation, cross-linking and texture – their relationship

The literature to date gives a clear indication that the texture of tofu can be changed with pre-determined preparation conditions. Without heating of soymilk, tofu is not formed due to the absence of hydrophobic interactions between proteins (Lee and Rha 1978, Liu 1997). Upon heating up to 97°C, the soy proteins are denatured. With thermal-denaturation, the –SH, S-S, and hydrophobic amino acid side chains, which were initially buried in native proteins, are exposed. The proteins are negatively charged due to the presence of charged amino acid residues such as glutamic and aspartic acid (–COO[–]). When a coagulant (CaSO₄·2H₂O) is added, the positive charge of Ca²⁺ ions neutralises the net charge of these proteins. As a result, protein molecules aggregate due

to the decrease of electrostatic repulsion. This allowed –SH/S-S interchange and hydrophobic interaction to occur inter- and intra-molecularly.

The hydrophobic interactions were reported to be dominant in this system (Sheard et al. 1986). Moreover, glycinin and β -conglycinin contain 39% and 41% w/w hydrophobic amino acid residues (Damodaran 1996). The glycinin and β -conglycinin fractions also contain free SH groups (Zayas 1997). With the combination of –SH/S-S interchange and hydrophobic interactions, soy proteins randomly aggregate. This leads to the formation of a three-dimensional network, which contributes to the microstructure of tofu (deMan et al. 1986, Fukushima 1980, Kohyama et al. 1995, Liu et al. 2004). By varying the water content, tofu texture can range from soft to firm (Liu 1997). The gelation mechanism of tofu is presented in Figure 1.11.

Glycinin and β -conglycinin are two major fractions of soy proteins, which have different denaturing temperatures. Glycinin and β -conglycinin denature at 85-95°C and 65-75°C, respectively. Upon boiling, the constituent sub-units of glycinin and β -conglycinin interact (Damodaran and Kinsella 1982, German et al. 1982, Renkema et al. 2001, Utsumi et al. 1984, Utsumi and Kinsella 1985). During boiling, β -conglycinin is firstly dissociated, followed by glycinin. The dissociated subunits of β -conglycinin unfold, aggregate and form a gel network within themselves until the glycinin has dissociated. Then, the dissociated subunits of glycinin associate with the network of β -conglycinin to form a larger macrocomplex with a finer and more orderly structure (Liu et al. 2004).

It was also reported that the ratio of glycinin to β -conglycinin influenced physical properties of tofu, such as the firmness (Guo and Ono 2005, Tay and Perera 2004). Although there are other interactions among soymilk components such as protein molecules, lipids, minerals and carbohydrates, the texture and structure of tofu are predominantly controlled by the protein-protein interactions. In a food system, the interaction of soy proteins with other constituents at a molecular level was found to be complex (Lakemond et al. 2000, Ono 2000).

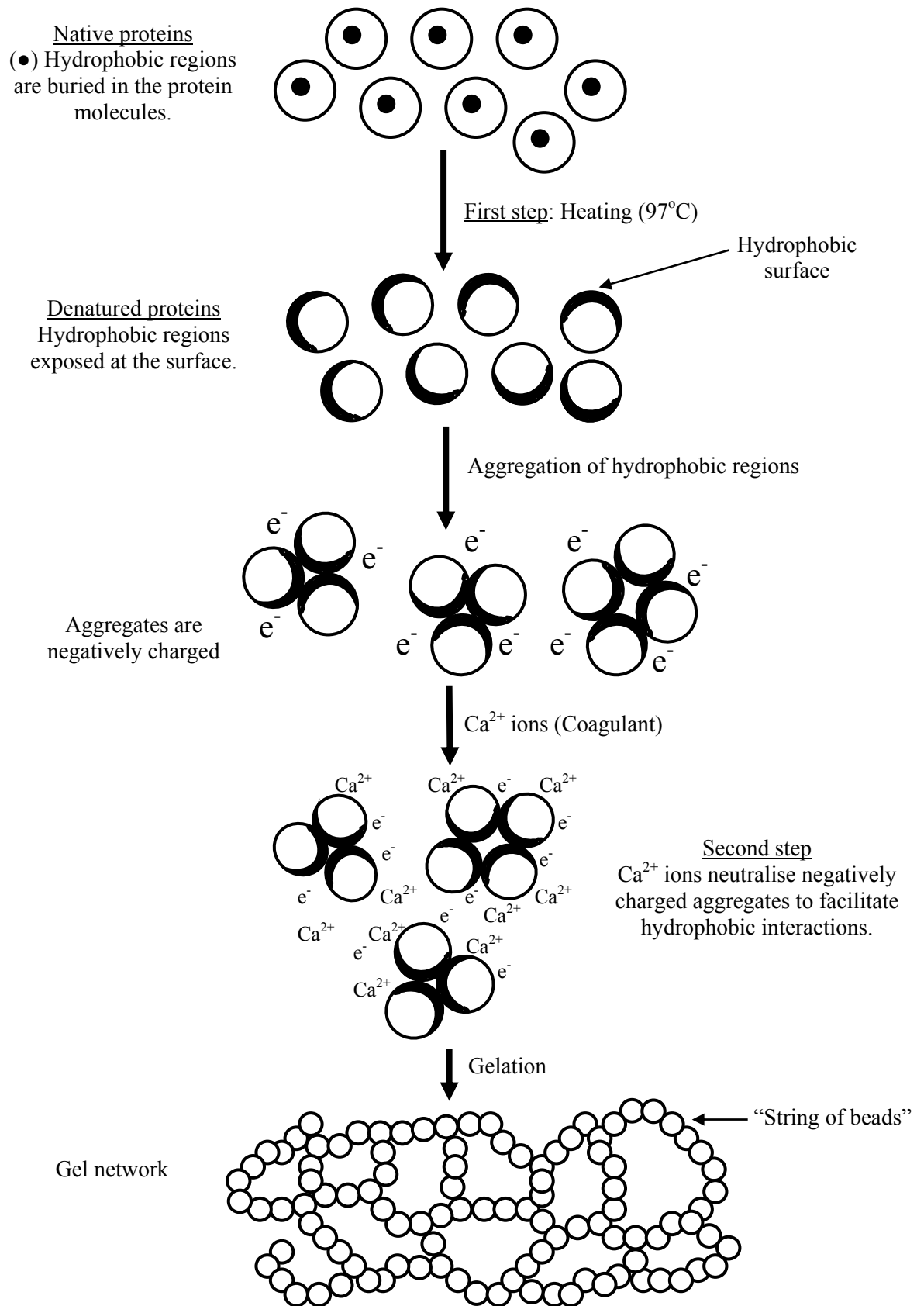


Figure 1.11: The gelation mechanism of tofu coagulated with CaSO_4 . (O) is the protein molecule. (●) is the hydrophobic region. Adapted from Kohyama et al. (1995).

Recent studies reported that a number of reactive compounds have been cross-linked with soy proteins to produce a desirable texture in soy products. For example, the cross-linking reaction induced by propylene glycol alginate resulted in a higher strength gel (Hua et al. 2003). It is believed that covalent cross-linking occurred between the polymeric aldehyde and soy proteins system. Thus it seemed likely that covalent cross-linking by carbonyl reagents during tofu making would produce tofu with altered texture.

TGA has previously been reported to introduce covalent cross-links in soy protein systems and modify their texture. For example, TGA has improved the tensile strength of biopolymers made from glycinin and whey protein isolate (Yildirim and Hettiarachchy 1998, Yildirim et al. 1996). The enzyme was also reported to enhance the quality of tofu made from old crops, providing a product with a firmer texture (Kuraishi et al. 2001). When a gel was prepared from soy protein isolate treated with TGA, the breaking strength and hardness increased. This was due to the formation of ϵ -(γ -glutamyl)lysyl isopeptide bonds, as a result of cross-linking in soy protein subunits (Nonaka et al. 1994). In the preparation of non-retort and retort tofu, the addition of TGA resulted in an increase in breaking strength (Soeda et al. 1995).

In view of the promising results obtained from a few soy protein products, this study aimed to further investigate the role of protein cross-linking on soy food texture, specifically tofu, by using the chemical reagents formaldehyde, glyceraldehyde and glutaraldehyde, and an enzyme, TGA.

Therefore, this thesis sets out to test the following hypotheses:

- i) In *in vitro* incubation, formaldehyde, glyceraldehyde and glutaraldehyde will cross-link soy proteins in a series of reactivity following the order glutaraldehyde > formaldehyde > glyceraldehyde. When applied *in situ*, these molecules will modify texture.
- ii) TGA will cross-link soy proteins *in vitro* and when applied *in situ*, it will alter the texture of tofu.
- iii) Chemical and enzymatic cross-linking in soy proteins will alter the fracturability and microstructure of tofu.

1.11 Thesis overview

In Chapter 2, the extraction of soy proteins by various literature methods is described. The characterisation of soy proteins was carried out using the technique of SDS-PAGE-densitometric analysis, reverse-phase high-performance liquid chromatography (RP-HPLC) and size-exclusion high-performance liquid chromatography (SE-HPLC).

Chapter 3 describes the impact of chemical reagents namely glutaraldehyde, formaldehyde and glyceraldehyde, on the soy proteins in defatted soy flour, glycinin and β -conglycinin during *in vitro* incubation. The reaction rates of these chemicals were evaluated, over a range of temperatures, as judged by SDS-PAGE and densitometric analysis. The participating subunits of these proteins were identified. The reaction rate of cross-linking was verified by SE-HPLC.

Chapter 4 describes the influence of TGA cross-linking on soy proteins in defatted soy flour, glycinin and β -conglycinin during *in vitro* incubation. The relative rates of reaction of TGA using various concentrations at room temperature were assessed by SDS-PAGE and densitometric analysis. The participating subunits of the soy proteins were identified.

Chapter 5 discusses the development of the method of tofu making and characterisation in the laboratory. Since there is no standard method of manufacturing tofu, it was necessary to standardise the procedure under controlled conditions. The testing conditions for textural properties were also established to minimize the variabilities of the Instron Universal Testing Machine (UTM).

Chapter 6 discusses the impact of glutaraldehyde and TGA added *in situ* on the textural properties of tofu at a range of concentrations. The resulting fracture force was then correlated with the analysis of soy proteins by SDS-PAGE and densitometric analysis.

Chapter 7 discusses the relationship between the microstructure and the fracturability of tofu. The microstructures were observed using scanning electron microscopy (SEM).

Chapter 8 summarises the study as a whole by comparing the chemical and enzymatic cross-linking of soy proteins. The introduction of glutaraldehyde or TGA into the tofu system is put forward as a tool to manipulate soy proteins to modify the microstructure, which then leads to changes of texture.

Chapter 9 reviews the materials and experimental procedures used in this thesis.

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CHAPTER 2

CHARACTERISATION

2.1 Background

Chapter 1 discussed the physicochemical properties of soy proteins and their potential to be manipulated through chemical and enzymatic cross-linking to change tofu texture. In this chapter, soy proteins are characterised prior to cross-linking experiments. This is important, because the composition of soy proteins, particularly the glycinin and β -conglycinin fractions, was reported to vary between soy bean varieties, which has a significant impact on the tofu textural properties (Bhardwaj et al. 1999, Min et al. 2005, Poysa and Woodrow 2002, Schaefer and Love 1992). Since glycinin and β -conglycinin fractions have different functional properties, several different methods have been developed to separate these two fractions.

Ultracentrifugation has been employed to characterise soy proteins on an analytical and preparative scale. This technique separates soy proteins into four fractions known as 2S, 7S, 11S and 15S globulins, based on their sedimentation coefficient values (Naismith 1955, Wolf and Briggs 1959). The estimated molecular weights were about 8-22, 180-210, 350 and 600 kDa and the approximate composition of 11S and 7S globulins was about 31% and 37% (Wolf and Cowan 1975). In separate studies, the approximate composition of 11S and 7S was 40% and 30% (Utsumi 1992, Utsumi et al. 1997). This shows that the composition of 11S and 7S globulins may vary. Based on their physicochemical properties, the solubilities and isoelectric points of the two fractions are different, enabling separation of the fractions on a commercial scale (Liu 1997).

On a commercial scale, defatted soy flakes are dissolved in aqueous solution (pH 8.0-9.0) at a temperature of 30-35°C. Then, the slurry is centrifuged to separate the solution from the solid material. Under these conditions, most of the soy proteins are dissolved in solution. The solution is then adjusted to pH 4.5 to precipitate the protein,

which contains both glycinin and β -conglycinin fractions. The precipitate is subsequently washed to remove non-protein materials, neutralised (pH 7) and spray-dried to obtain a protein isolate. The protein content in the isolate is about 90% (Liu 1997). Recently, electroacidification and ultrafiltration methods have been used to produce soy protein isolate and concentrate (Bezinet et al. 1998, Mondor et al. 2004).

However, for work on a small scale, glycinin and β -conglycinin are independently isolated from soy proteins for further characterisation. Glycinin and β -conglycinin are different in their compositions and structures, and consequently, they possess differing functionalities (Yamauchi et al. 1991). They also have different isoelectric points, allowing them to be isolated by varying the pH of the solution. For instance, both glycinin and β -conglycinin can be isolated using a dilute Tris buffer (0.03–0.06 M). At a pH ranging from 6.1 to 6.6, most of the glycinin precipitates, whilst β -conglycinin remains soluble. When the pH of the remaining Tris buffer is reduced to between 4.0 and 5.6, β -conglycinin precipitates concomitantly with glycinin, because glycinin precipitates over the pH range 4.4 to 6.8 (Thanh and Shibasaki 1976b).

Although success in isolating glycinin and β -conglycinin depends on the precise extraction conditions, most studies report the use of isoelectric precipitation for optimum isolation of either glycinin or β -conglycinin (Koshiyama 1972, Thanh et al. 1975, Hill and Breidenbach 1974). In view of these promising results, four published methods were selected for initial screening.

This chapter explains how these methods were assessed before choosing the most appropriate one to isolate glycinin and β -conglycinin sequentially. Once the method had been chosen, this was followed by characterisation using three analytical techniques, namely: i) SDS-PAGE, ii) densitometric analysis, iii) Reverse-Phase High Performance Liquid Chromatography (RP-HPLC), and Size-Exclusion High-Performance Liquid Chromatography. The discussion highlights the quantification of glycinin and β -conglycinin produced using the chosen method. Finally, the reproducibility of the chosen method is discussed.

2.2 Extraction and quantification

2.2.1 Choosing a method of extraction

In earlier studies, several methods have been reported to isolate glycinin and β -conglycinin, based on their isoelectric points, by varying the pH. Among them, four methods were chosen for initial screening in order to select the most appropriate method to isolate glycinin and β -conglycinin fractions. The four methods were those of Scilingo and Anon (1996), Peterson and Wolf (1988), Thanh et al. (1975) and Ji et al. (1999). They are schematically presented in Figure 2.1, 2.2, 2.3 and 2.4 respectively. These respective methods will be referred to as the Scilingo, Peterson, Thanh and Ji methods in the following discussion.

In this study, the isolation was initiated with an isoelectric precipitation, by varying the pH of the medium, to isolate the glycinin and β -conglycinin fractions sequentially from the soy proteins. To select the best method, glycinin and β -conglycinin fractions from these four extraction methods were assessed by SDS-PAGE. The visual assessment was based on the intensities of major subunits present in each fraction. For glycinin, the subunits of interest are A and B, whilst for β -conglycinin, the subunits of interest are of α' , α and β . After selection, this method was repeated several times to determine its reproducibility.

The resulting SDS-PAGE gel is shown in Figure 2.5. Lanes 1-4 represent the glycinin fraction from the four methods. Comparing the glycinin intensities in these lanes, the glycinin fractions from Peterson method (Lane 2) yielded the highest quantity of the glycinin fraction. The Scilingo (Lane 1) and Thanh (Lane 3) methods separated glycinin from β -conglycinin as well, but the intensities of the A and B subunits were lower than those from the Peterson method, indicating that lesser quantities of glycinin were extracted with the Scilingo and Thanh methods. The Ji method (Lane 4) yields significantly less glycinin than the other three methods. In all four extraction methods, there was a cross-contamination from β -conglycinin subunits, but they were at a significantly lower level than the glycinin fractions, with similar intensities in each case.

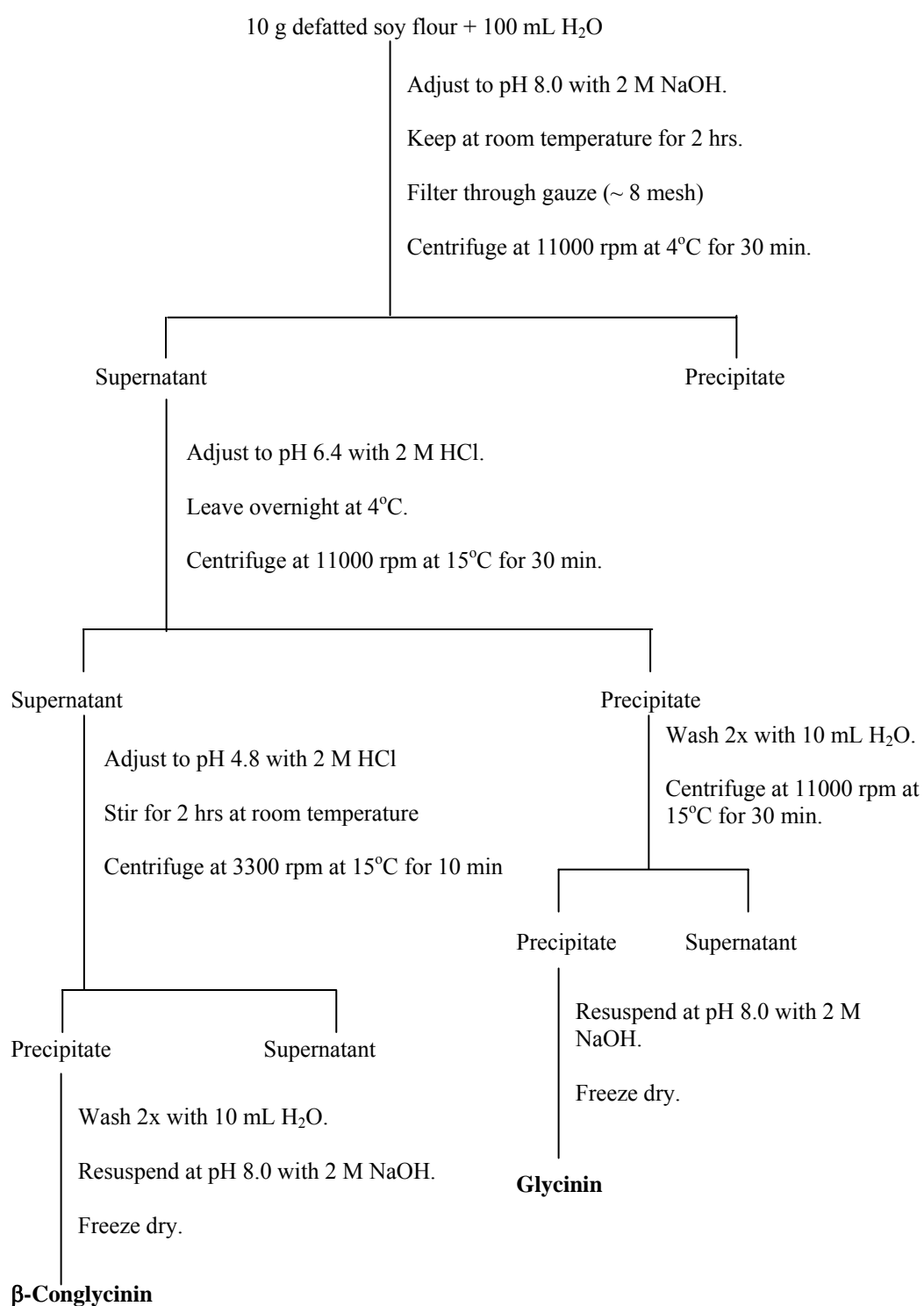


Figure 2.1: Schematic outline of the Scilingo method of sequential isolation of glycinin and β-conglycinin from defatted soy flour. Adapted from Scilingo and Anon (1996).

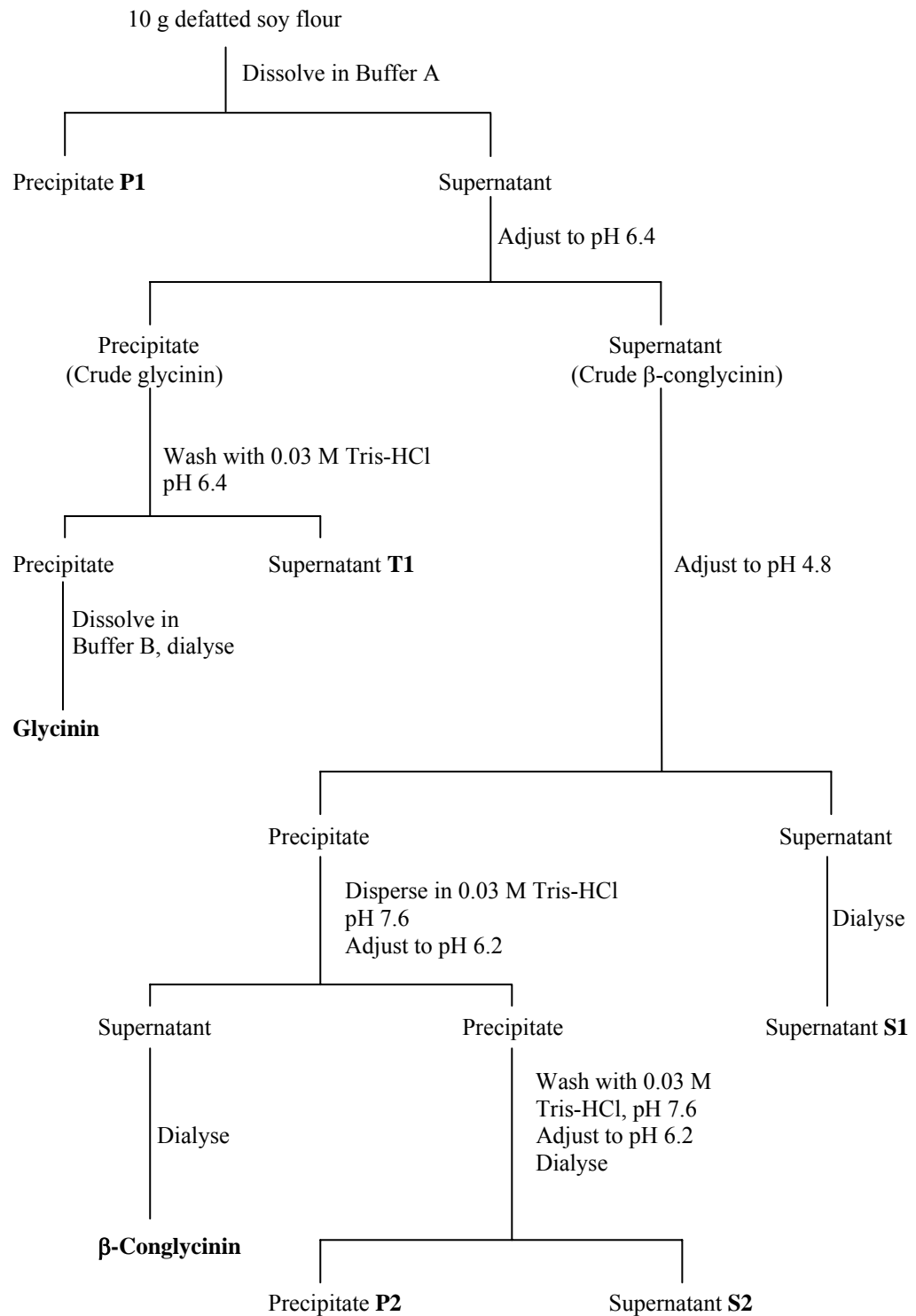


Figure 2.2: Schematic outline of Peterson method of sequential isolation of glycinin and β -conglycinin from defatted soy flour. Buffer A = 0.03 M Tris-HCl, pH 8.0, 10 mM 2-mercaptoethanol; Buffer B = 0.4 M NaCl, 33 mM K_2HPO_4 , 2.6 mM KH_2PO_4 , 0.02 % NaN_3 , 10 mM 2-mercaptoethanol, pH 7.6. Adapted from Peterson and Wolf (1988).

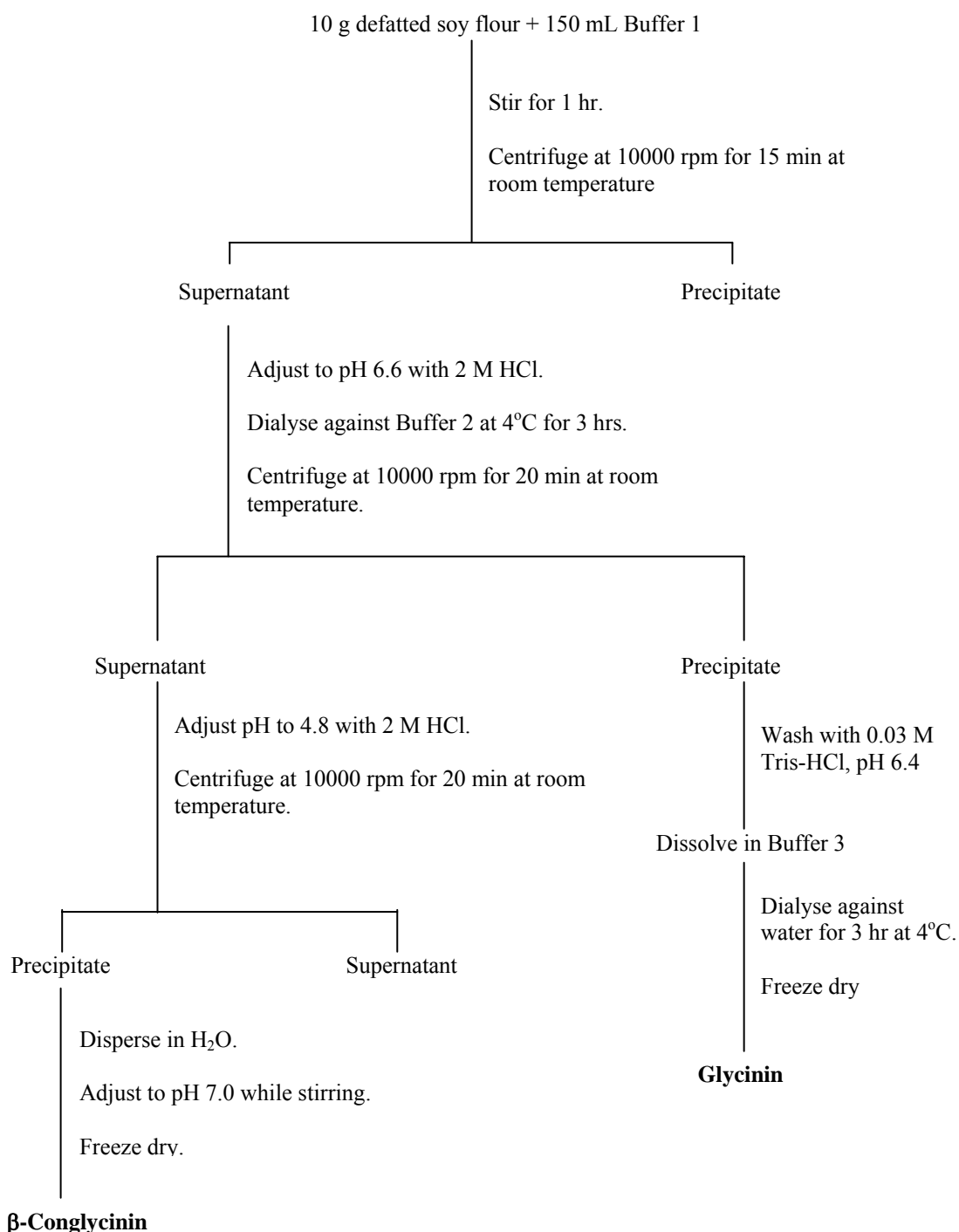


Figure 2.3: Schematic outline of the Thanh method of sequential isolation of glycinin and β-conglycinin from defatted soy flour. Adapted from Thanh et al. (1975).

Buffer 1: 63 mM Tris-HCl containing 10 mM 2-mercaptoethanol, pH 7.8.

Buffer 2: 63 mM Tris-HCl containing 10 mM 2-mercaptoethanol, pH 6.6 at 4°C.

Buffer 3 = 0.4 M NaCl, 33 mM K₂HPO₄, 2.6 mM KH₂PO₄, 0.02 % NaN₃, 10 mM 2-mercaptoethanol, pH 7.6.

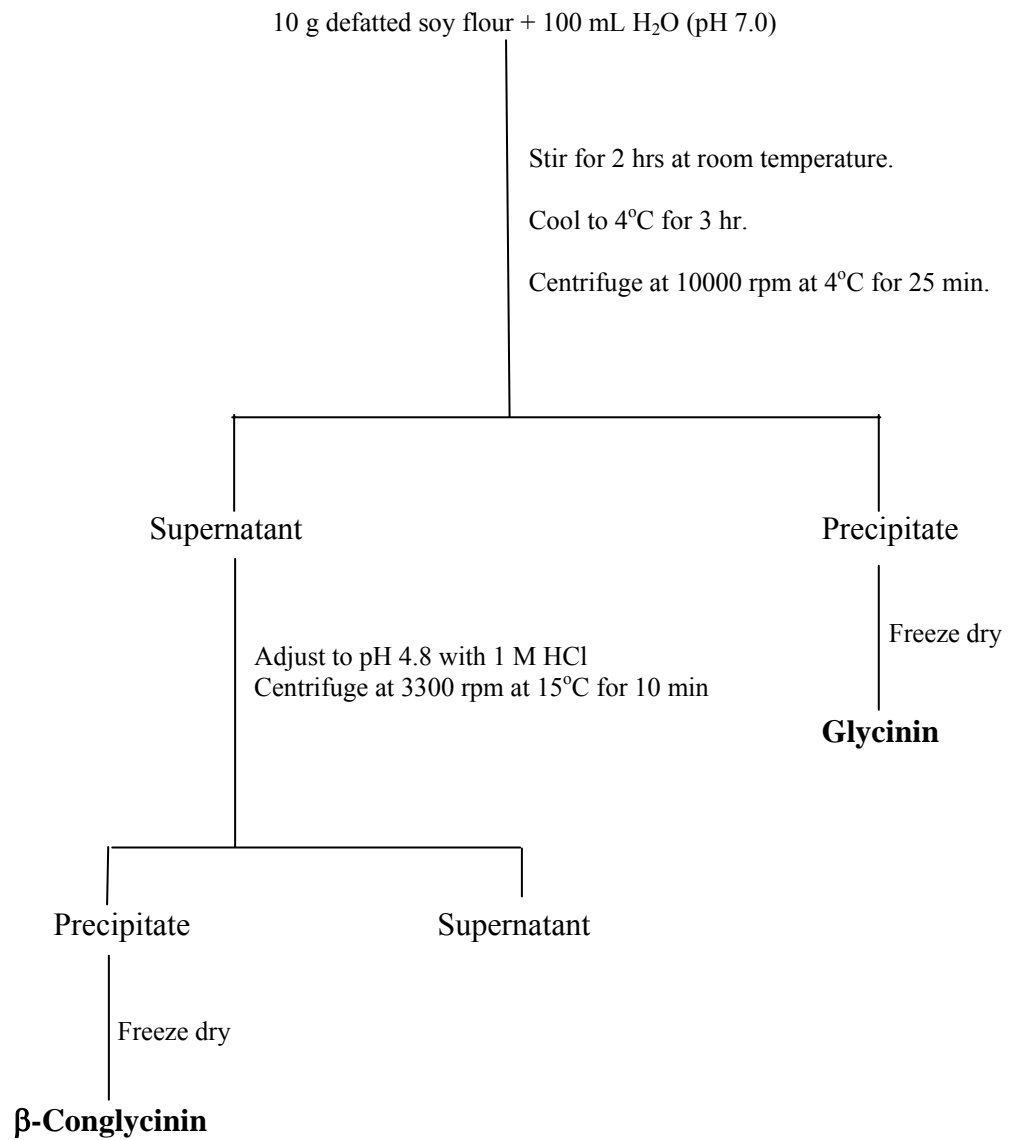


Figure 2.4: Schematic outline of the Ji method of sequential isolation of glycinin and β -conglycinin from defatted soy flour. Adapted from Ji et al. (1999).

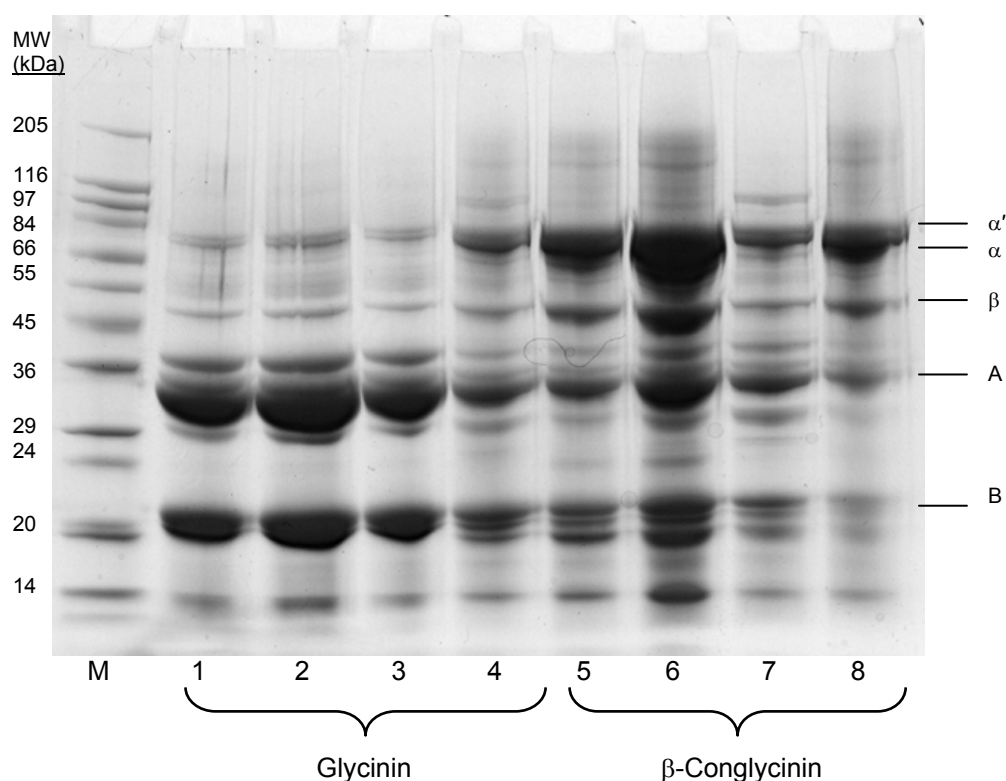


Figure 2.5: The SDS-PAGE profiles of glycinin and β -conglycinin from the four methods of extraction. M = Marker; Lane 1-4 = glycinin; Lane 5-8 = β -conglycinin; 1 and 5 = Scilingo method; 2 and 6 = Peterson method; 3 and 7 = Thanh method; 4 and 8 = Ji method; A and B = Acidic and Basic subunits of glycinin; α' , α and β = subunits of β -conglycinin. In each case, 2 mg of sample was dissolved in 250 μ L of water. Then 20 μ L solution was added with 20 μ L treatment buffer and subsequently 40 μ L sample was loaded onto the gel (4-20% gradient). The gel is representative of two replicate gels of two replicate extractions.

Lane 5 to 8 represents the β -conglycinin fractions from four methods of extraction. Comparing the band intensities, the Peterson method (Lane 6) has the highest intensity, particularly of α' , α and β subunits, compared to the other three methods, suggesting that an optimum amount of β -conglycinin was obtained. This method also showed a certain degree of cross-contamination from the glycinin fraction. The Thanh method (Lane 7) contained the lowest quantities of α' , α and β subunits and also a certain degree of cross-contamination of glycinin. Although the Scilingo (Lane 5) and Ji (Lane 8) method yielded similar amount of α' , α and β subunits, their quantities were less than from the Peterson method (Lane 6). Therefore, based on the SDS-PAGE profiles, the method of Peterson and Wolf (1988) was chosen as the method of

extraction for all subsequent work because of its ability to separate optimum amount of glycinin and β -conglycinin fraction.

2.2.2 Quantification

Having identified the most promising method of extracting glycinin and β -conglycinin using the Peterson method, as schematically presented in Figure 2.2, the amounts of glycinin and β -conglycinin and other fractions from this method were quantified. On each fraction, the protein content was also determined. Details on how these fractions were obtained for characterisation are presented in Section 9.2.2 Chapter 9.

After being freeze-dried, the mass of the material in each dialysed fraction was recorded. The protein concentration in each fraction was also determined, using the Bradford method (Bradford 1976). These data are presented in Table 2.1. On average, a total mass of protein and non-protein materials recovered from defatted soy flour was about 6.46 g from 10 g of defatted soy flour (65%). The P1 fraction yielded the greatest quantity of material, accounting for 3.39 g (34%). Glycinin and β -conglycinin fractions accounted for about 0.76 g (7.6%) and 0.6 g (6%), respectively. The other fractions, T1, S1, S2 and P2 were in a range of 1.5 g (1.5%) to 9.3 g (9.3%). A total mass of proteins recovered from the defatted soy flour was about 1.56 g (30%) from 5.2 g proteins.

The protein yield (1.56 g) obtained by this method was far lower than the yield obtained from a combination method of isoelectric precipitation and ultracentrifugation. (Thanh and Shibasaki 1976b) obtained 42.6 g total proteins per 100 g of defatted soybean meal by using this combination method. However, the higher amount of glycinin obtained (0.76 g) than β -conglycinin (0.60 g) was consistent with the earlier studies (Iwabuchi and Yamauchi 1987, Thanh and Shibasaki 1976b).

Table 2.1: The mass and concentration of fractions extracted from defatted soy flour with Peterson method (see Figure 2.4).

Fraction	Mass (g)	Mass Percentage ¹ (%)	Protein content ² (%)	Protein mass (g)
Defatted soy flour (Starting material)	10	100	52.0 ³	5.20 ³
<u>Fractions</u>				
Glycinin	0.76 ± 0.01	7.6 ± 0.1	92.0 ± 1.4 ²	0.70 ± 0.01
β-Conglycinin	0.60 ± 0.05	6.0 ± 0.05	85.0 ± 1.0 ²	0.51 ± 0.04
P1	3.39 ± 0.19	33.9 ± 1.9	2.7 ± 0.3 ²	0.09 ± 0.01
T1	0.34 ± 0.01	3.4 ± 0.1	29.5 ± 2.4 ²	0.10 ± 0.01
S1	0.93 ± 0.13	9.3 ± 1.3	10.0 ± 1.9 ²	0.09 ± 0.01
S2	0.15 ± 0.01	1.5 ± 0.1	43.7 ± 9.6 ²	0.07 ± 0.01
P2	0.29 ± 0.01	2.9 ± 0.1	0.0 ± 0.0 ²	-
Total mass	6.46 ± 0.06	64.6 ± 0.06	24.3 ± 0.7 ²	1.56 ± 0.04

The values are the means ± standard error of the means from triplicate extractions.

¹The percentage is based on the mass of defatted soy flour (10 g).

²Protein content was determined using the Bradford method (Bradford 1976).

³Protein content was determined using a LECO instrument (Chapter 9).

P1 = Precipitate of defatted soy flour after dissolving in Buffer A.

T1 = Residual supernatant after washing crude glycinin with 0.03 M Tris-HCl at pH 6.4.

S1 = Residual supernatant after precipitation from crude β-conglycinin at pH 4.8.

S2 = Residual supernatant after washing polymerized β-conglycinin with 0.03 M Tris-HCl at pH 7.6 and readjusting to pH 6.2.

P2 = Precipitate of polymerized β-conglycinin after washing with 0.03 M Tris-HCl at pH 7.6 and readjusting to pH 6.2.

It is believed that the loss of 35% total material or 55% of total protein was due to the dialysing of protein. The dialysis tubing used had 12-14 kDa cut-off, thus allowing only >14 kDa proteins to be retained in the tubing. Since the soy protein contained 22% of the 2S fraction, which had a molecular weight ranging from 8 to 21 kDa (Table 1.1 Chapter 1), lower molecular weight proteins at < 14 kDa were lost. SDS-PAGE profiles in Figure 2.6 clearly showed that proteins with molecular weight lower than 14 kDa

were almost absent all fractions (Lane 2-8). However, the method was more convenient than alternative methods with higher yields.

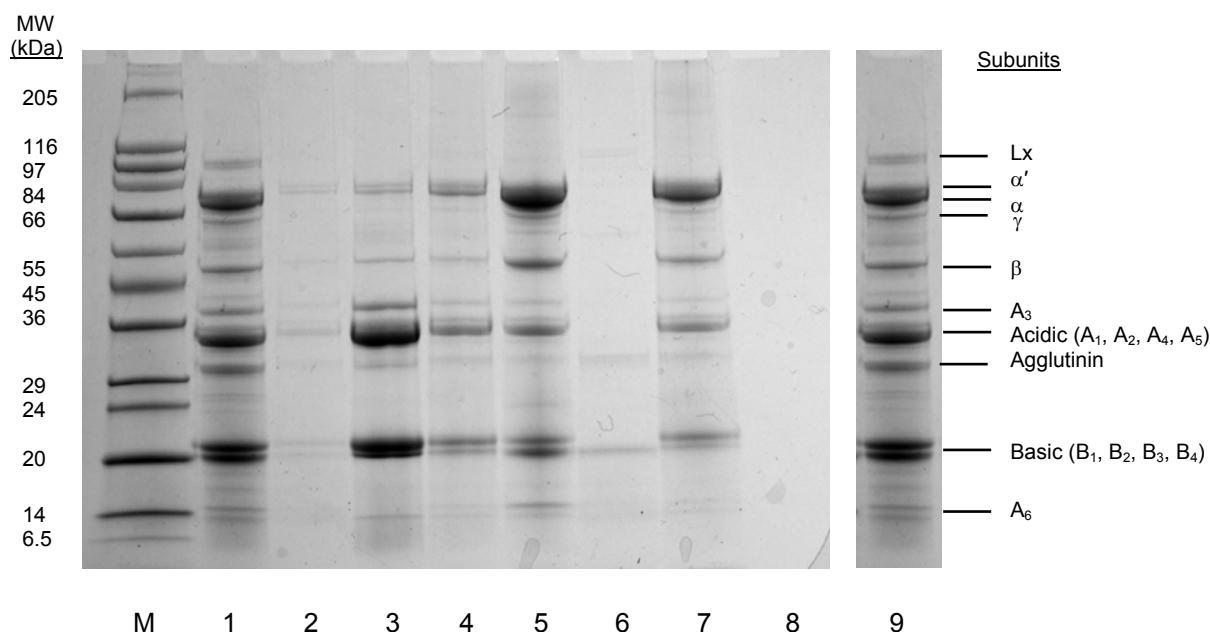


Figure 2.6: The SDS-PAGE profiles of fractions extracted from defatted soy flour. M = standard marker; Lane 1 = defatted soy flour (starting material); Lane 2 = the precipitate of defatted soy flour after dissolving in Buffer A (P1); Lane 3 = the glycinin fraction; Lane 4 = the residual supernatant after washing crude glycinin with 0.03 M Tris-HCl at pH 6.4 (T1); Lane 5 = the β -conglycinin fraction; Lane 6 = the residual supernatant after precipitation from crude β -conglycinin at pH 4.8 (S1); Lane 7 = the residual supernatant after washing polymerized β -conglycinin with 0.03 M Tris-HCl at pH 7.6 and readjusting to pH 6.2 (S2); Lane 8 = the precipitate of polymerized β -conglycinin after washing with 0.03 M Tris-HCl at pH 7.6 and readjusting to pH 6.2 (P2); Lane 9 = defatted soy flour labelling all subunits present in soy proteins; Lx = Lipoxygenase, α' , α , β and γ are the subunits of β -conglycinin; A and B are the acidic and basic subunits of glycinin. For defatted soy flour (Lane 1 and 9), 3 mg sample was dissolved in 500 μ L water, and for other extracts (Lane 2 to 8), 1 mg sample was dissolved in 500 μ L water. From each solution, an equal amount of sample solution (20 μ L) and treatment buffer (20 μ L) were added, and finally 20 μ L sample was loaded onto the gel in each case. This gel is representative of triplicate gels of triplicate extractions.

The protein content in each fraction was also examined using the Bradford method (Bradford 1976). Protein content in glycinin was 92%, higher than that of the β -conglycinin (85%). These results compare well with earlier work, where similar findings were reported (Table 2.2). Differences in soybean cultivars have been found to

contribute to the respective protein content of glycinin and β -conglycinin (Ji et al. 1999, Khatib et al. 2002, Riblett et al. 2001). Thus, regardless of the methods employed or the various cultivars used, the protein content of glycinin was found to be consistently higher than that of β -conglycinin, with the exception of the Macon cultivar in the Riblett study.

Table 2.2: Comparison of protein content in glycinin and β -conglycinin from different methods of extraction.

Method/Soybean cultivars	Glycinin (%)	β -Conglycinin (%)
Peterson method (This study)* Commercial defatted soy flour (Sigma)	92.0 ± 1.4	85.0 ± 1.0
Iwabuchi and Yamauchi (1987)	85	55
<u>Ji et al. (1999)</u> Proto Vinton Sturdy	94.6 ± 2.1 95.6 ± 0.4 88.0 ± 1.0	71.1 ± 2.8 76.9 ± 1.6 71.7 ± 0.6
<u>Khatib et al. (2002)</u> K1430 Hutcheson K93-90-29 KS4997	91.89 ± 0.10 93.25 ± 0.11 91.01 ± 0.14 89.89 ± 0.06	88.31 ± 0.03 84.63 ± 0.25 90.17 ± 0.06 85.06 ± 0.09
Kohyama and Nishinari (1993)	95	92
<u>Riblett et al. (2001)</u> Enrei IL2 Macon Ohio FG1	87.11 ± 1.98 96.70 ± 0.50 78.87 ± 0.87 93.41 ± 1.70	78.61 ± 0.81 73.04 ± 1.91 88.90 ± 2.15 83.95 ± 1.25
Wu et al. (1999)	90.3	71.3
Wu et al. (2000)	92.8	62.6

*The values are the means \pm standard error of the means from triplicate extractions.

The lower protein content in the β -conglycinin fraction is due to the presence of carbohydrates, since β -conglycinin is a glycoprotein (Thanh and Shibasaki 1976a). Khatib et al. (2002) reported that the carbohydrate in the β -conglycinin fraction was in the range of 1.8% to 8.8% in various cultivars. Pernollet and Mosse (1983) found that β -conglycinin contained 4% to 5% carbohydrate. In addition to being a glycoprotein, Kitabatake et al. (1990) and Sessa (1993) reported that β -conglycinin showed a strong affinity to carbohydrate that might be dependent on soybean genotype. The protein content of the other fractions, P1, T1, S1 and S2 was about 3%, 30%, 10% and 44% respectively. The P2 fraction showed no protein content.

2.3 SDS-PAGE profiles

SDS-PAGE has been widely used for characterisation of soy proteins for various purposes, including variety identification in breeding programs, and for molecular weight and compositional estimation (Ji et al. 1999, Mujoo et al. 2003, Riblett et al. 2001, Wu et al. 2000, Zarkadas et al. 1997). In this study, SDS-PAGE was used to characterise the protein fractions in term of protein subunits, molecular weight, protein partitioning within the fractions and cross-contamination. Moreover, SDS-PAGE profiles facilitate densitometric analysis in order to quantify protein subunits. An SDS-PAGE gel illustrating the protein profiles of the extracts is presented in Figure 2.6.

Defatted soy flour contained all protein subunits (Lane 1, 9). The order of estimated molecular weight was consistent with those results reported in earlier studies (Fontes et al. 1984, Mujoo et al. 2003, Nishinari et al. 1991, Petruccelli and Anon 1995).

As expected, Lane 3 shows glycinin fraction, which was comprised of A and B subunits as described in previous literature (Fontes et al. 1984, Mujoo et al. 2003, Nishinari et al. 1991, Petruccelli and Anon 1995). A subunits consisted of A₁, A₂, A₃, A₄, A₅ and A₆ polypeptides, whilst B subunits consist of B₁, B₂, B₃ and B₄ polypeptides (Nishinari et al. 1991, Petruccelli and Anon 1995). The value of molecular weight is based on the approximate value observed on the gel. The A₃ polypeptide had the highest

molecular weight at nearly 36 kDa, followed by a group of acidic (A) subunits (A_1 , A_2 , A_4 , A_5) at about 30 kDa. Near 29 kDa, a protein band of agglutinin was observed (Nishinari et al. 1991, Petruccelli and Anon 1995). Next, a group of B subunits (B_1 , B_2 , B_3 and B_4) at nearly 20 kDa molecular weight, and lastly the A_6 polypeptide had the lowest molecular weight at about 14 kDa (Nishinari et al. 1991, Petruccelli and Anon 1995). These molecular weights of A and B were slightly different than previous studies. Peng et al. (1984) reported the acidic and basic subunits were in a range of 37-45 and 18-20 kDa, whilst Yaklich (2001) found the acidic and basic subunits were in a range of 34-44 and 20 kDa.

Meanwhile, β -conglycinin shows of α' , α and β subunits, which had an estimated molecular weight at about 80, 75 and 50 kDa respectively, in agreement with Mujoo et al. (2003). However, these results were slightly different than the results found by Yamauchi et al. (1991), in which α' , α and β subunits were in a range of 57-72, 57-68 and 45-52 kDa. Within the β -conglycinin subunits, the α' and α subunits were found to be at a relatively higher intensity than the β subunit. Differences in molecular weights of α' , α and β subunits have been reported due to the differences in soybean varieties. Yaklich (2001) reported that α' , α and β subunits of β -conglycinin were about 76, 72 and 53 kDa respectively, whereas Hirano et al. (1987) reported even lower molecular weights of α' , α and β subunits at about 58, 57 and 42 kDa respectively.

A faint band of γ -conglycinin was also observed near the α' and α subunits. The γ -conglycinin has no similarities with β -conglycinin and is a minor protein among soybean proteins (Hirano et al. 1987, Yamauchi et al. 1985). Although there was no varietal type stated in the commercial defatted soy flour (Sigma) used for this study, the molecular weight profile of the subunits on the SDS-PAGE gel was consistent with most earlier reports (Fontes et al. 1984, Mujoo et al. 2003, Nishinari et al. 1991, Petruccelli and Anon 1995).

Other than the glycinin and β -conglycinin fractions, the other SDS-PAGE profiles were from the remaining extracts derived from defatted soy flour. The P1 fraction was the undissolved materials from defatted soy flour, and, as such, this profile showed only faint protein bands observed on the gel, confirming that both glycinin and

β -conglycinin fractions were mostly dissolved in the extraction buffer (Buffer A) at pH 8.0 (Mondor et al. 2004). T1 was the residual supernatant after washing crude glycinin with 0.03 M Tris-HCl at pH 6.4. This washing step at pH 6.4 extracts the residual β -conglycinin fraction remaining in the crude glycinin. The SDS-PAGE profile shows that the T1 fraction comprised α' , α , β , acidic (A) and basic (B) subunits, with a band intensity less than that of the glycinin. The corresponding protein content was estimated at about 30% (Table 2.1).

The fractions of S1, S2 and P2 are the residual fractions after extraction of crude β -conglycinin. S1 was the residual supernatant after crude β -conglycinin had been precipitated at pH 4.8 and showed only faint bands. Thus, with the absence of β -conglycinin bands in the S1 fraction, most of the β -conglycinin had precipitated.

The P2 fraction showed no proteins band on the SDS-PAGE gel, indicating that no β -conglycinin fraction was contained in this precipitate. This precipitate was obtained from twice washing precipitates of supernatant S1 with Tris-HCl buffer at pH 7.6, and then readjusted to pH 6.2. The resulting supernatant after this washing was the S2 fraction. On the SDS-PAGE gel, the S2 fraction showed a β -conglycinin profile. The protein content in the S2 fraction was about 44%. This suggests that a certain amount of β -conglycinin fraction has remained in the S2 fraction after the second wash, but had a lower protein content (44%) than the β -conglycinin fraction (85%).

Cross-contamination: The sequential extraction of glycinin and β -conglycinin by varying pH invariably results in cross-contamination, with the degree of contamination dependent on the particular extraction method employed (Eldridge and Wolf 1967, Hill and Breidenbach 1974, Koshiyama 1972). As expected, the Peterson and Wolf (1988) method adopted in this research showed cross-contamination. As judged by the SDS-PAGE profiles (Figure 2.6), glycinin was contaminated to a lesser degree than the β -conglycinin. Contaminating acidic (A) and basic (B) subunits of glycinin were clearly observed in the β -conglycinin fraction, whereas contaminating β -conglycinin subunits were only faintly visible in the glycinin. For this study, obtaining higher purity was not a major concern, since the focus was the impact of cross-linking reagents and the reactivity could still be monitored by SDS-PAGE profiling.

2.4 Densitometric analysis

To estimate the percentage of each of the subunits present in the proteins, densitometric analysis was carried out on the defatted soy flour, glycinin and β -conglycinin fractions. The densitograms of defatted soy flour, glycinin and β -conglycinin are presented in Figure 2.7. The patterns observed were in agreement with results reported earlier on the characterisation of soybeans (Kitamura et al. 1976, Nishinari et al. 1991, Petruccelli and Anon 1995, Yagasaki et al. 1997). The composition of each subunit corresponds with those subunits detected in SDS-PAGE and are presented in Table 2.3. The following discussion refers to Figure 2.7 and Table 2.3 concurrently.

The densitogram of defatted soy flour (Figure 2.7A) consisted of all peaks of unfractionated soy proteins. Based on the densitogram, the composition of glycinin was about 53%, whilst the composition of β -conglycinin was about 29% (Table 2.3). In the glycinin fraction, the acidic (A) and basic (B) subunits composed about 39% and 24% respectively. In the β -conglycinin fraction, α' , α and β subunits made up about 7%, 14% and 8% of the total protein, respectively. Murphy and Resurreccion (1984) reported that the composition of glycinin and β -conglycinin were in the range of 42-57% and 15-21% respectively. Conversely, the amount of β -conglycinin can be higher than glycinin, depending on the environmental conditions and genetic materials (Saio et al. 1969, Wolf 1961, Wolf et al. 1962).

The densitogram of glycinin (Figure 2.7B) consisted of two major groups of peaks: i) a group of A subunits consisting of $A_{1,2,4,5}$ polypeptides, and ii) a group of B subunits, consisting of B_{1-4} polypeptides. The minor peaks are A_3 and A_6 polypeptides and are acidic subunits. A cross-contamination of α' , α and β subunits from the β -conglycinin fraction was also observed. As shown in Table 2.3, the amount of glycinin in the fraction accounted for about 81%, whilst 10% was contaminants from β -conglycinin subunits. Within the glycinin fraction, the A subunits accounted for about 43% and the B subunits accounted for about 38%.

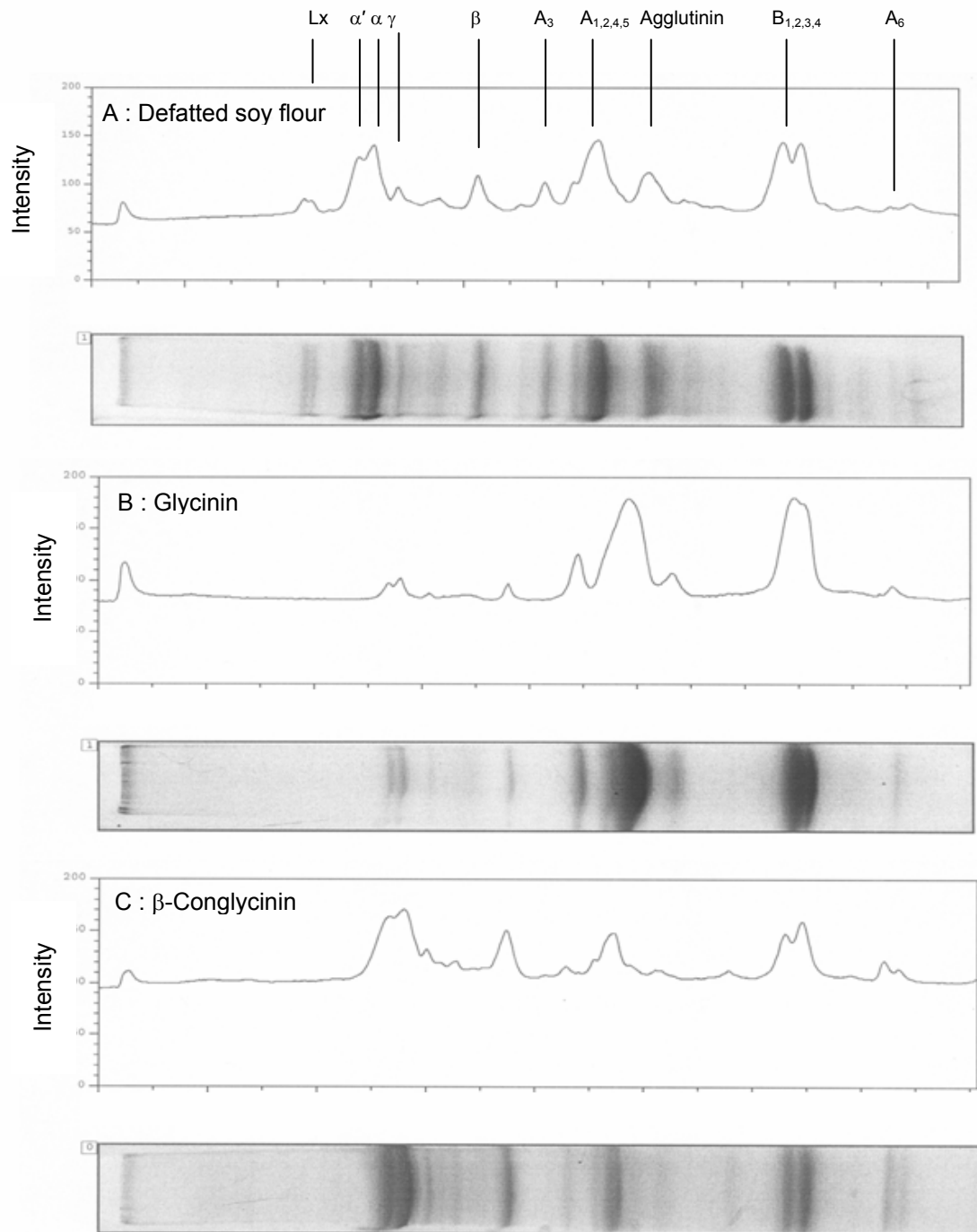


Figure 2.7: The densitogram from SDS-PAGE profiles of defatted soy flour (A), glycinin (B) and β -conglycinin (C). The subunits profiles on the SDS-PAGE gel are designated on the corresponding peaks of the densitogram. The densitograms are representatives of triplicate extractions.

Table 2.3: The composition of subunits in defatted soy flour, glycinin and β -conglycinin from densitometric analysis.

Sample	*Subunits (%)									
	Others			β -Conglycinin			Glycinin			
	Lx	γ	Agglutinin	α'	α	β	A ₃	A _{1-2,4-4}	A ₆	B ₁₋₄
Defatted soy flour	5.10 \pm 0.22	5.20 \pm 0.05	7.590 \pm 0.19	7.38 \pm 0.01	13.65 \pm 0.90	7.80 \pm 1.45	5.59 \pm 0.31	23.02 \pm 0.86	0.95 \pm 0.47	23.73 \pm 0.56
Glycinin	-	1.91 \pm 0.37	7.53 \pm 0.40	3.56 \pm 0.70	3.19 \pm 2.77	3.30 \pm 0.67	11.24 \pm 1.16	28.86 \pm 2.15	2.53 \pm 0.55	37.88 \pm 3.37
β -Conglycinin	-	7.69 \pm 1.27	3.23 \pm 0.06	12.14 \pm 0.46	21.74 \pm 0.86	11.53 \pm 0.47	3.57 \pm 0.26	15.42 \pm 2.04	3.39 \pm 0.07	21.27 \pm 0.30

Lx = Lipoxxygenase; A = Acidic; B = Basic; *Values are the means \pm standard error of the means from triplicate measurements.

The densitogram of β -conglycinin (Figure 2.7C) consisted of four prominent groups of peaks: i) a group of α' and α , ii) β subunit, iii) A subunits of glycinin, and iv) B subunits of glycinin. As shown in Table 2.3, the β -conglycinin fraction accounted for about 45% and the remaining 44% was the glycinin contaminants in the β -conglycinin fraction. Within the β -conglycinin fraction, the α' , α and β subunits accounted for about 12%, 22% and 11% respectively, whereas the A and B subunits accounted for about 23% and 21% respectively.

2.5 RP-HPLC analysis

In principle, RP-HPLC separates proteins based on the hydrophobic interaction between the proteins, the stationary phase, and the mobile phase during elution (Bietz 1983). RP-HPLC has been reported to be a most successful method in separating several seed proteins, such as wheat proteins (Burnouf and Bietz 1989, Sutton et al. 1989), barley, rye and oat proteins (Wieser and Belitz 1989), corn proteins (Paulis and Bietz 1988), and sorghum proteins (Sastry et al. 1986). This technique has major advantages, such as profiling the surface hydrophobicity of seed proteins, and has been employed to characterise soy proteins. For example, RP-HPLC has been reported to be useful in screening soybean cultivars by determining the differences in the RP-HPLC chromatogram of glycinin and β -conglycinin fractions (Buehler et al. 1989, Mujoo et al. 2003, Oomah et al. 1994, Riblett et al. 2001). Other uses include separation of β -conglycinin, γ -conglycinin and B subunits of glycinin from soy proteins (Hirano et al. 1987). Peterson and Wolf (1988) separated individual subunits of glycinin for analytical and preparative purposes. This technique has also been reported to be capable of detecting the presence of soy proteins in a mixture of beef-soy protein blends (Ashoor and Stiles 1987).

The RP-HPLC chromatograms of defatted soy flour, glycinin and β -conglycinin are presented in Figure 2.8, which shows a number of peaks eluted from the column. Since peak resolution is based on the surface hydrophobicity of protein, the peak profiles do not correlate with those found in SDS-PAGE profiles.

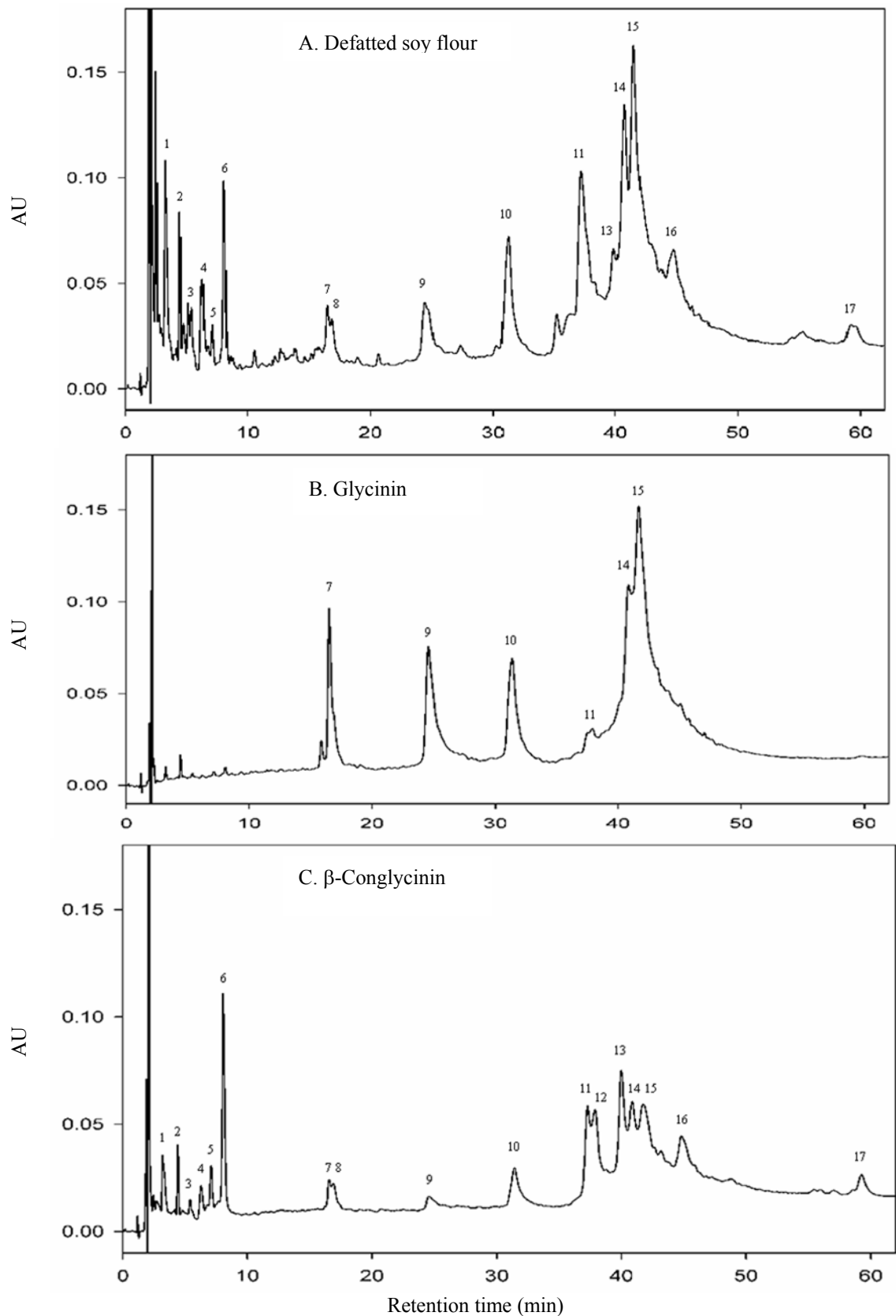


Figure 2.8: RP-HPLC chromatograms of protein in defatted soy flour (A) and extracted glycinin (B) and β -conglycinin (C) fractions using the Peterson method. The chromatograms are representatives of triplicate chromatograms of three extractions.

The chromatogram of defatted soy flour (Figure 2.8A) showed resolution of at least 17 major peaks that belonged to the glycinin and β -conglycinin fractions. In comparison, glycinin (Figure 2.8B) showed resolution of four major peaks, all eluting after 15 min retention time. In the β -conglycinin (Figure 2.8C) chromatogram, six peaks of β -conglycinin eluted earlier (0-10 min retention time) and were followed by contaminant peaks of glycinin.

According to Peterson and Wolf (1988), the elution of peaks began with β -conglycinin fraction, followed by A and B subunits of glycinin, confirming that β -conglycinin fraction had higher surface hydrophobicities, whilst B subunits had the least. Although the individual peaks are not assigned to the subunits of glycinin and β -conglycinin, in general, the patterns of peak elution can be used to distinguish between the glycinin and β -conglycinin fractions.

The elution of β -conglycinin before glycinin was similar to results reported earlier (Kato et al. 1987, Yamauchi et al. 1985, Yuno et al. 1988). Dissimilarities were found in the number of resolved peaks, which are thought to be related to the soybean varieties (Buehler et al. 1989, Mujoo et al. 2003, Oomah et al. 1994, Riblett et al. 2001). The compositions of peak areas of glycinin and β -conglycinin were calculated as presented in Table 2.4.

2.6 SE-HPLC

SE-HPLC has emerged as a rapid and precise method of characterising a number of plant proteins and their fractions including soy proteins (El Nour et al. 1998, Oomah et al. 1994, Singh and MacRitchie 2004). In addition to characterisation using SDS-PAGE and RP-HPLC, SE-HPLC has been employed to quantitate the soy proteins that are associated with particular soybean cultivars or soy flour quality (Oomah et al. 1994).

Table 2.4: The compositions of peaks in defatted soy flour, glycinin and β -conglycinin chromatograms.

Peak No.	Glycinin (%)	β -Conglycinin (%)
1	n.d.	2.8 ± 0.2
2	n.d.	1.7 ± 0.1
3	n.d.	0.6 ± 0.1
4	n.d.	1.5 ± 0.2
5	n.d.	1.8 ± 0.2
6	n.d.	9.1 ± 0.6
7	9.5 ± 0.2	2.4 ± 0.2
8	n.d.	2.1 ± 0.2
9	15.0 ± 0.6	2.7 ± 0.2
10	12.2 ± 0.5	6.0 ± 0.2
11	1.9 ± 0.0	8.9 ± 0.7
12	n.d.	8.6 ± 0.1
13	n.d.	13.5 ± 0.4
14	21.1 ± 1.6	12.4 ± 0.4
15	40.2 ± 0.8	16.6 ± 0.9
16	n.d.	6.7 ± 0.4
17	n.d.	2.5 ± 0.1

Values are the means \pm standard error of the means from three chromatograms of triplicate extractions. n.d. = non detectable.

Recently, SE-HPLC has been employed to monitor the polymerisation of protein fractions and changes in molecular size distribution with time caused by heating, TGA, or dehydroascorbic acid (Fayle and Gerrard 2002, Fayle et al. 2000, Gerrard et al. 2001, Rasiah 2002, Singh and MacRitchie 2004). Since SE-HPLC is able to monitor the loss of proteins during cross-linking, the kinetics of cross-linking can be evaluated.

SE-HPLC was employed to assess the reproducibility of the extraction method. Therefore, only glycinin and β -conglycinin fractions were used. The distributions of SE-HPLC traces of glycinin and β -conglycinin fractions are presented in Figures 2.9 and 2.10, respectively. Figure 2.9 showed that the glycinin fraction eluted four protein peaks, denoted as peaks A, B, C and D. Since the separation is based on the molecular size or hydrodynamic volume of a molecule (Welling and Welling-Wester 1998), peak

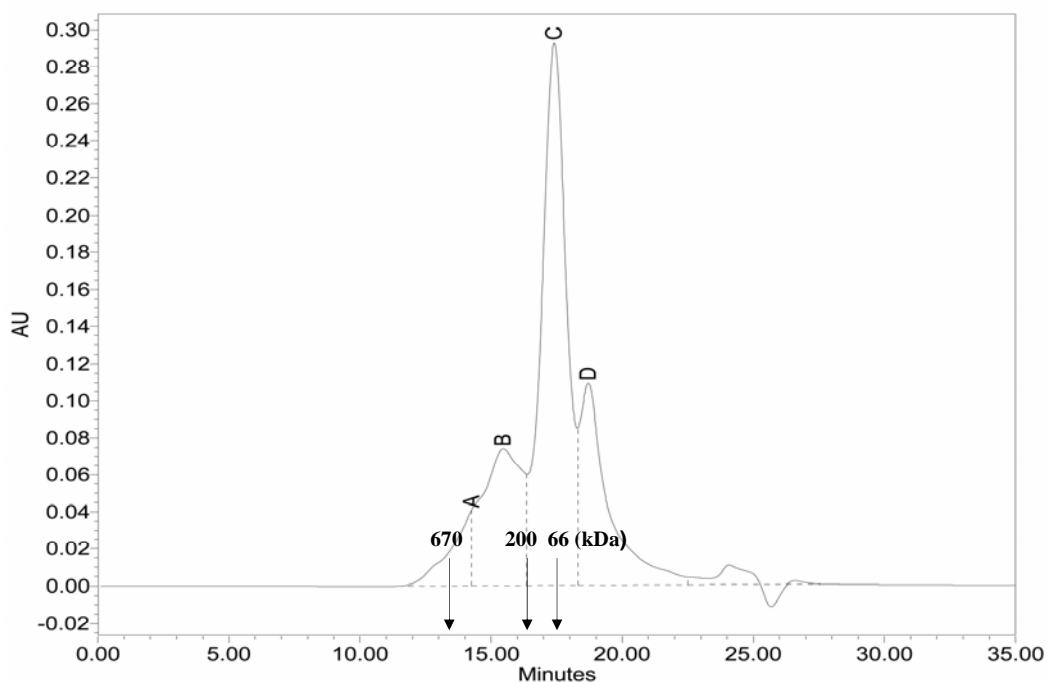


Figure 2.9: A typical SE-HPLC traces of glycinin. Peaks A, B, C and D represent glycinin monomers. These traces represent triplicate chromatograms of triplicate extractions.

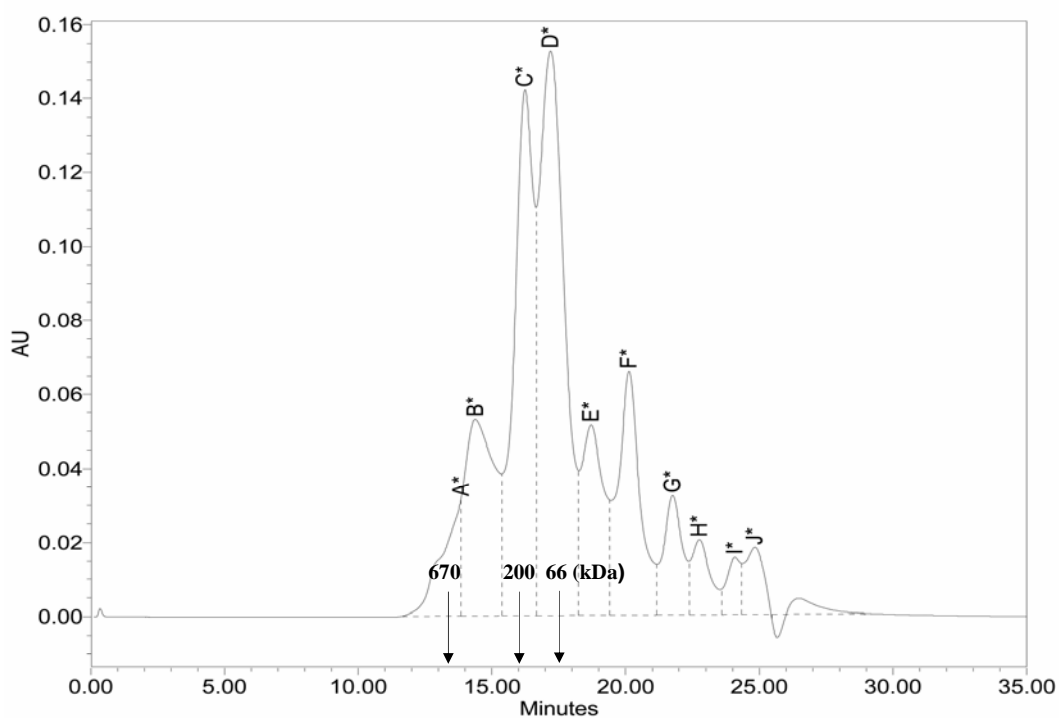


Figure 2.10: A typical SE-HPLC traces of β -conglycinin. Peaks A*-J* represent β -conglycinin monomers. These traces represent triplicate chromatograms of triplicate extractions.

A represent the highest molecular size, followed by B, C and D. The relative quantities of these peaks are presented in Table 2.5. Figure 2.10 showed that the β -conglycinin fraction eluted ten protein peaks denoted as peaks A*-J*. Peak A* represents the highest molecular size, whilst J* was the lowest. The relative quantities of these peaks are presented in Table 2.6. During cross-linking, the loss of these proteins will be quantified to obtain the kinetics of the reaction as discussed in section 3.3 Chapter 3.

Table 2.5: The composition of peaks of glycinin traces from SE-HPLC.

Peak	Composition ^a (%)
A	6.5 \pm 0.1
B	21.1 \pm 0.5
C	49.6 \pm 1.1
D	22.8 \pm 0.6

^aValues are the means \pm standard errors of the mean of triplicate measurements of total protein.

Table 2.6: The composition of peaks of β -conglycinin traces from SE-HPLC.

Peak	Composition ^a (%)
A*	4.4 \pm 0.2
B*	12.0 \pm 0.1
C*	22.5 \pm 0.8
D*	28.3 \pm 0.9
E*	8.2 \pm 0.6
F*	11.8 \pm 0.5
G*	5.1 \pm 0.3
H*	3.1 \pm 0.1
I*	2.0 \pm 0.3
J*	2.6 \pm 0.1

^aValues are the means \pm standard errors of the mean of triplicate measurements of total protein.

2.7 Conclusion

Among the four extraction methods examined, the method of Peterson and Wolf (1988) has been selected to extract glycinin and β -conglycinin fractions from defatted soy flour. This method was found to extract the most glycinin and β -conglycinin, when compared to the other three methods. Moreover, the protein content in glycinin and β -conglycinin was found to be about 92% and 85% respectively, which compared favourably with previous studies.

For assessing the efficiency and reliability of the method of extraction, SDS-PAGE, densitometric analysis, RP-HPLC and SE-HPLC techniques were used. The SDS-PAGE profile confirmed the composition and molecular masses of all subunits in soy proteins. RP-HPLC showed the degree of surface hydrophobicity of the soy proteins, whilst SE-HPLC separated by size. However, a certain degree of cross-contamination was found in the glycinin and β -conglycinin fractions, as observed on the SDS-PAGE gel. The cross-contamination and purity of glycinin and β -conglycinin were not unexpected, due to the fraction overlap in isoelectric point. Thus, the glycinin and β -conglycinin fractions have been fully characterised prior to the cross-linking experiment described in Chapters 3 and 4.

2.8 References

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CHAPTER 3

CROSS-LINKING OF SOY PROTEINS BY GLUTARALDEHYDE, FORMALDEHYDE AND GLYCERALDEHYDE

3.1. Background

The main objective of these experiments was to determine the reaction rate of the cross-linking reaction in each treatment, under different conditions, as a function of incubation time. Defatted soy flour, glycinin and β -conglycinin fractions extracted using the Peterson and Wolf (1988) method (Chapter 2) were employed in cross-linking studies with glutaraldehyde, formaldehyde and glyceraldehyde at a range of concentrations and temperatures. To study the rate of reaction, two techniques were employed: i) SDS-PAGE with densitometric analysis, and ii) SE-HPLC analysis. These techniques provide a quantitative measurement of protein cross-linking and were described in Chapter 2.

3.2. SDS-PAGE analysis of cross-linking reactions

Earlier studies have reported that glutaraldehyde, formaldehyde and glyceraldehyde are capable of cross-linking a range of proteins, as summarised in Table 3.1. The cross-linking reaction yielded dimers, trimers or aggregated proteins, depending on the temperature and concentration of the carbonyl groups, as demonstrated by SDS-PAGE and SE-HPLC (Fayle 1998, Rasiah 2002). Reactivity was dependent on the molecular structure of the cross-linking reagents (Gerrard et al. 2002, Gerrard et al. 2003b). Silva et al. (2004) revealed that when 0.25% glutaraldehyde cross-linked bovine serum albumin or casein, the average molecular mass increased to 20-fold and 40-fold that of the native structure, respectively. However, little is known about the reactions of these three molecules with soy proteins.

Table 3.1: Cross-linking reactions using glutaraldehyde, formaldehyde and glyceraldehyde with a range of proteins from various studies.

Study	Carbonyl group	Protein
Acharya et al. (1988)	Glyceraldehyde	Ribonuclease
Galembeck et al. (1977)	Formaldehyde	Lysozyme, Ribonuclease
Gerrard et al. (2002)	Glutaraldehyde, Formaldehyde, Glyceraldehyde	Ribonuclease
Gerrard et al. (2003b)	Glutaraldehyde, Formaldehyde, Glyceraldehyde	Wheat proteins
Marquie (2001), Marquie et al. (1997)	Glutaraldehyde, Formaldehyde	Cottonseed protein
Meade et al. (2003)	Glutaraldehyde	Ribonuclease
Silva et al. (2004)	Glutaraldehyde	Bovine albumin, Casein

Defatted soy flour, and the glycinin and β -conglycinin fractions characterised in Chapter 2 were cross-linked with glutaraldehyde, formaldehyde and glyceraldehyde and analysed by SDS-PAGE and SE-HPLC. Earlier studies had reported that glutaraldehyde was more reactive than formaldehyde (Gerrard et al. 2002). Following this research, the concentration of glutaraldehyde was fixed at 1 and 2 mM, lower than for formaldehyde and glyceraldehyde. The incubation temperatures for glutaraldehyde were fixed at 20, 30, 40 and 50°C.

Since formaldehyde and glyceraldehyde were far less reactive, a higher concentration was needed (Gerrard et al. 2003a, Gerrard et al. 2003b). To achieve an equivalent level of cross-linking as glutaraldehyde, 50 and 100 mM were fixed, after preliminary examinations using lower concentrations. Two different incubation temperatures, 30 and 50°C were employed to compare the cross-linking reactivity. The experimental design is presented in Table 3.2. Details of sample preparation are described in section 9.3.1 (Chapter 9).

Table 3.2: The conditions employed to incubate soy proteins with cross-linking reagents. Parameters were fixed after several preliminary investigations.

Sample	Cross-linking reagents and concentrations	Temp (°C)	Incubation period (hr)	Sampling interval (hr)
Defatted soy flour Glycinin β -Conglycinin	1 mM glutaraldehyde	20	12	2
		30	6	1
		40	3	0.5
		50	1	0.25
	2 mM glutaraldehyde	20	6	1
		30	3	0.5
		40	1.5	0.25
		50	0.75	0.125
	50 mM formaldehyde	30	6	1
		50	3	0.5
	100 mM formaldehyde	30	3	0.5
		50	1.5	0.25
	50 mM glyceraldehyde	30	6	1
		50	3	0.5
	100 mM glyceraldehyde	30	3	0.5
		50	1.5	0.25

All experiments were carried out in duplicate. 6 mg of defatted soy flour, 2 mg each of glycinin and β -conglycinin were dissolved in 600 μ L of distilled water respectively. An appropriate volume of cross-linking reagent was added and the final volume was made up to 1 mL with distilled water.

Longer incubation periods and sampling times in the formaldehyde and glyceraldehyde treatments were implemented in order to obtain equivalent SDS-PAGE profiles as glutaraldehyde treatment and facilitate densitometric analysis. For each of the cross-linking reagent, doubling the concentration at fixed temperature, the incubation period was shortened by half.

Densitometric analysis on SDS-PAGE was performed to monitor the changes of protein band intensity as a function of time. After converting band intensity into protein concentration, the protein concentration (mg/mL) versus incubation time was plotted. In all cases, the curves fitted a logarithmic decay curve. The curves were then fitted with a first-order kinetic equation for a linear relationship (Equation 1).

$$\ln c = \ln c_0 - kt \quad (\text{Equation 1})$$

where c is protein concentration at time t , c_0 is protein concentration at time 0, t is incubation time and k is the rate constant, subsequently used as a measure of reaction rate.

In order to simplify the analysis, the quantification was carried out on all subunits in defatted soy flour, glycinin and β -conglycinin as defined earlier in Chapter 2. The remaining protein was quantified and plotted as a function of time, fitting the first-order kinetic equation to obtain the rate constant. Finally, the rate of loss of protein was estimated for each treatment.

3.2.1 Defatted soy flour

SDS-PAGE: The incubation of defatted soy flour with glutaraldehyde, formaldehyde and glyceraldehyde resulted in varying degrees of cross-linking. As expected, glutaraldehyde was found to be the most reactive, followed by formaldehyde, with the least reactive being glyceraldehyde. Typical SDS-PAGE profiles are presented in Figures 3.1, 3.2 and 3.3. The relative concentration of formaldehyde and glyceraldehyde used in these incubations was 50-fold higher than glutaraldehyde concentration in order to achieve similar effect as for lower concentrations of glutaraldehyde. All other variables were the same across the three incubations. The intensities of control samples were unchanged throughout incubation in all treatments.

In the glutaraldehyde incubation (Figure 3.1), the intensities of the α' , α , β , A and B subunits were gradually reduced, and at the same time, the aggregates and multimeric proteins (Multimer a) are formed, as evidenced by a smearing towards the top of the gel. This indicates that most of the protein subunits were cross-linked. A

similar effect was observed during the formaldehyde experiment (Figure 3.2) at a higher concentration. Thus, glutaraldehyde was much more reactive than formaldehyde. The cross-linking with glyceraldehyde resulted in very slow cross-linking. The formation of the multimeric proteins (Multimer *a*) proceeded at the slowest rate (Figure 3.3). Therefore, glyceraldehyde was the least reactive cross-linking reagent of the three molecules employed.

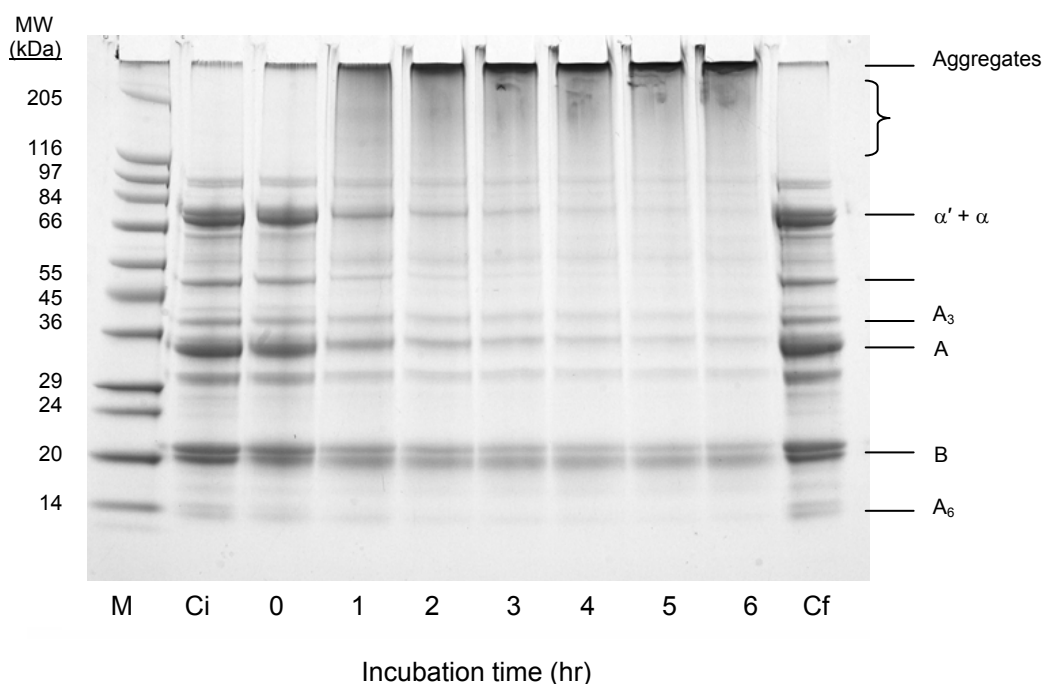


Figure 3.1: A typical SDS-PAGE profile of defatted soy flour incubated with 1 mM glutaraldehyde. Incubation condition: 6 mg of defatted soy flour was incubated with 1 mM glutaraldehyde at 30°C in 1 mL solution. M = Marker; Ci = Control at initial incubation; Cf = Control at final incubation time. The image is a representative of duplicate gels.

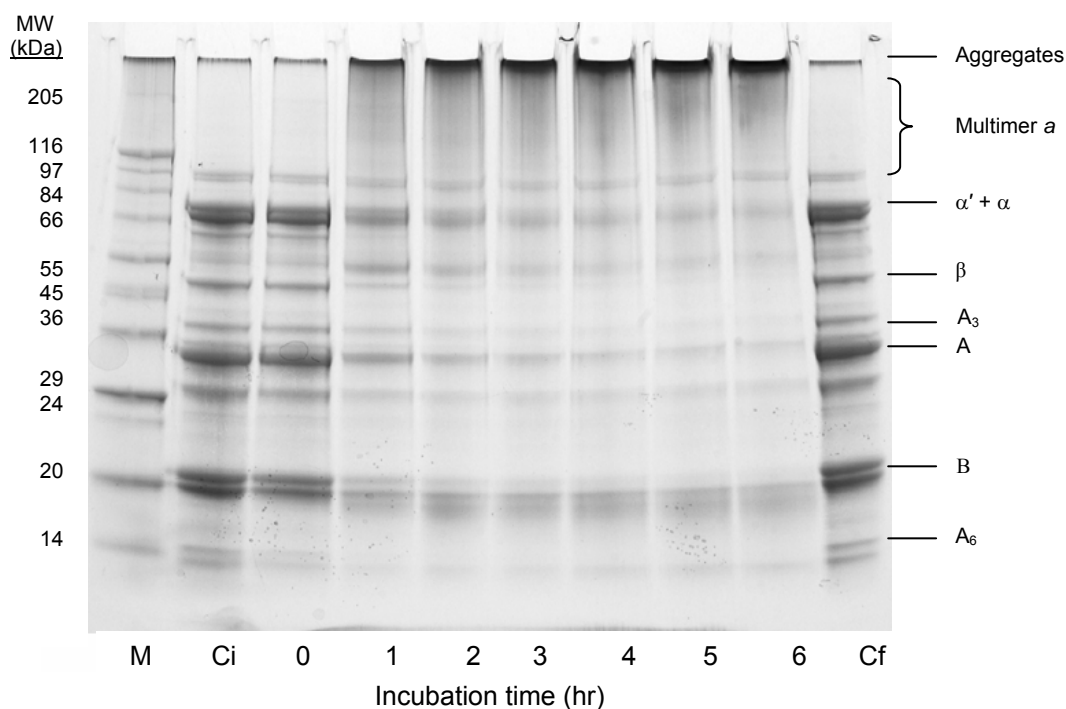


Figure 3.2: A typical SDS-PAGE profile of defatted soy flour incubated with 50 mM formaldehyde. Incubation condition: 6 mg of defatted soy flour was incubated with 50 mM formaldehyde at 30°C in 1 mL solution. M = Marker; Ci = Control at initial incubation; Cf = Control at final incubation time. The image is a representative of duplicate gels.

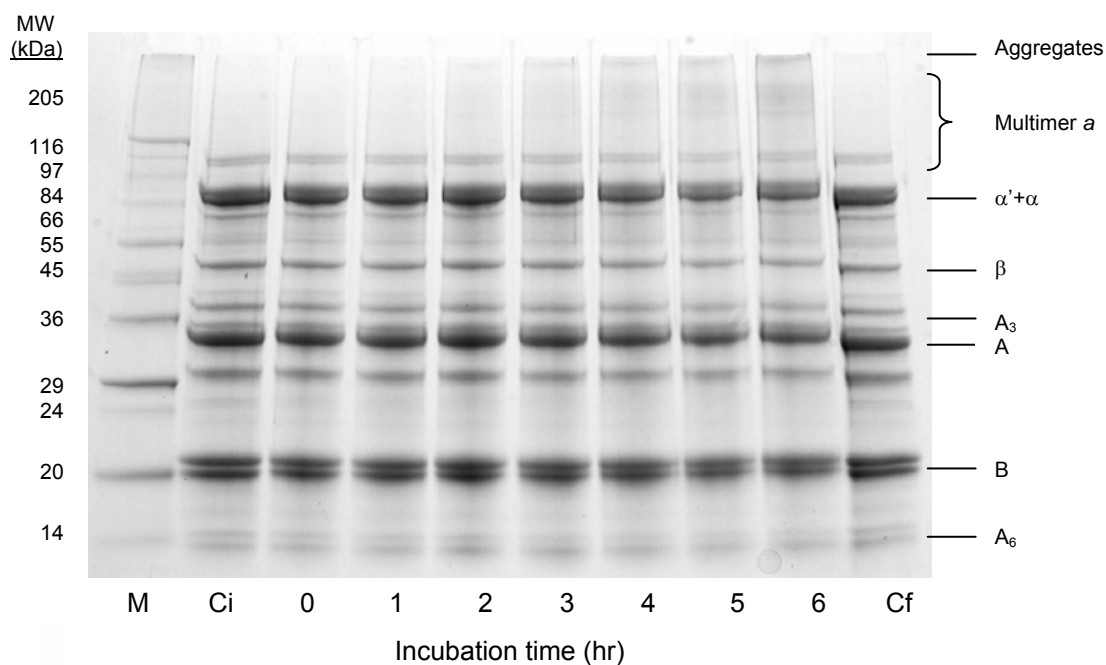


Figure 3.3: A typical SDS-PAGE profile of defatted soy flour incubated with 50 mM glyceraldehyde. Incubation condition: 6 mg of defatted soy flour was incubated with 50 mM glyceraldehyde at 30°C in 1 mL solution. M = Marker; Ci = Control at initial

incubation; Cf = Control at final incubation time. The image is a representative of duplicate gels.

Remaining protein: Defatted soy flour was not totally dissolved in water. In the preliminary examination using water, only 40% of the proteins dissolved in water. Thus, the estimation of proteins was based on the 40% proteins of defatted soy flour. The remaining protein in the solution was estimated using densitometry on SDS-PAGE gel. The remaining protein was calculated based on the intensity of all subunits of soy proteins as defined earlier. The general pattern of protein loss during incubation of defatted soy flour with cross-linking reagents at a range of temperatures showed an exponential decay, which was fitted to the natural logarithm of the first-order kinetic equation, Equation 1 (Section 3.2). A typical linear curve is presented in Figure 3.4. Comparing the slopes from a range of temperatures showed that a higher temperature resulted in a higher cross-linking rate.

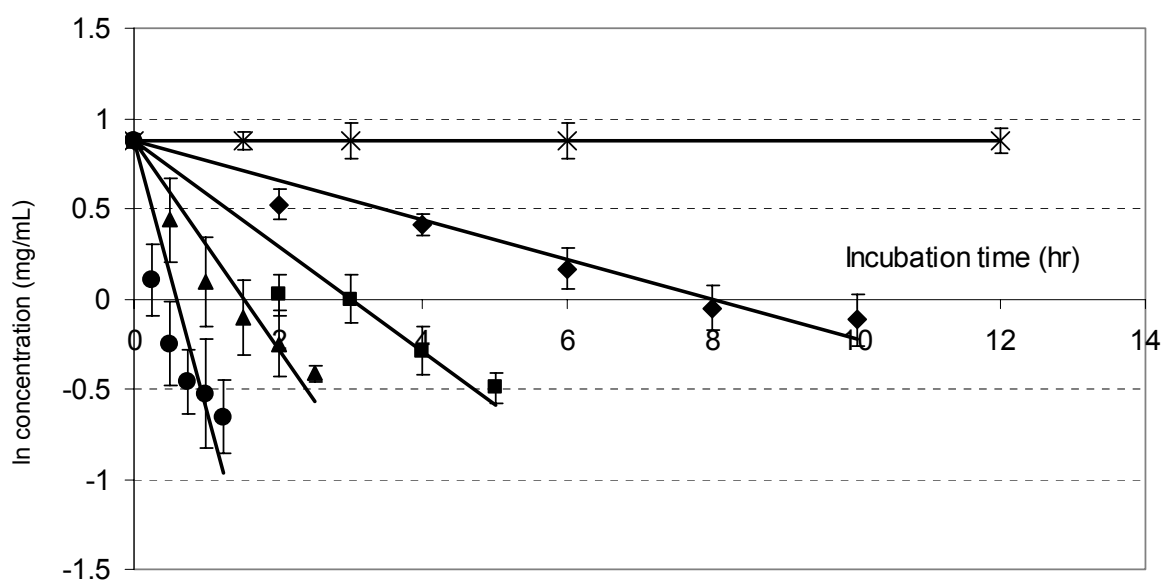


Figure 3.4: Typical patterns of linear relationships of protein in defatted soy flour during incubation. Slope of these lines were used as a measure of the rate of protein loss for all incubations. Values are the means of duplicate measurements. Error bars represent the standard error of the means. Key:- x = control; ♦ = 20°C; ■ = 30°C; ▲ = 40°C; ● = 50°C.

Comparison of reaction rates: The linear equations show a good fit to the data. In most cases, the slope of linear equation, which represents the reaction rate, increased with the increment of temperature and concentration as shown in Figure 3.5. This is

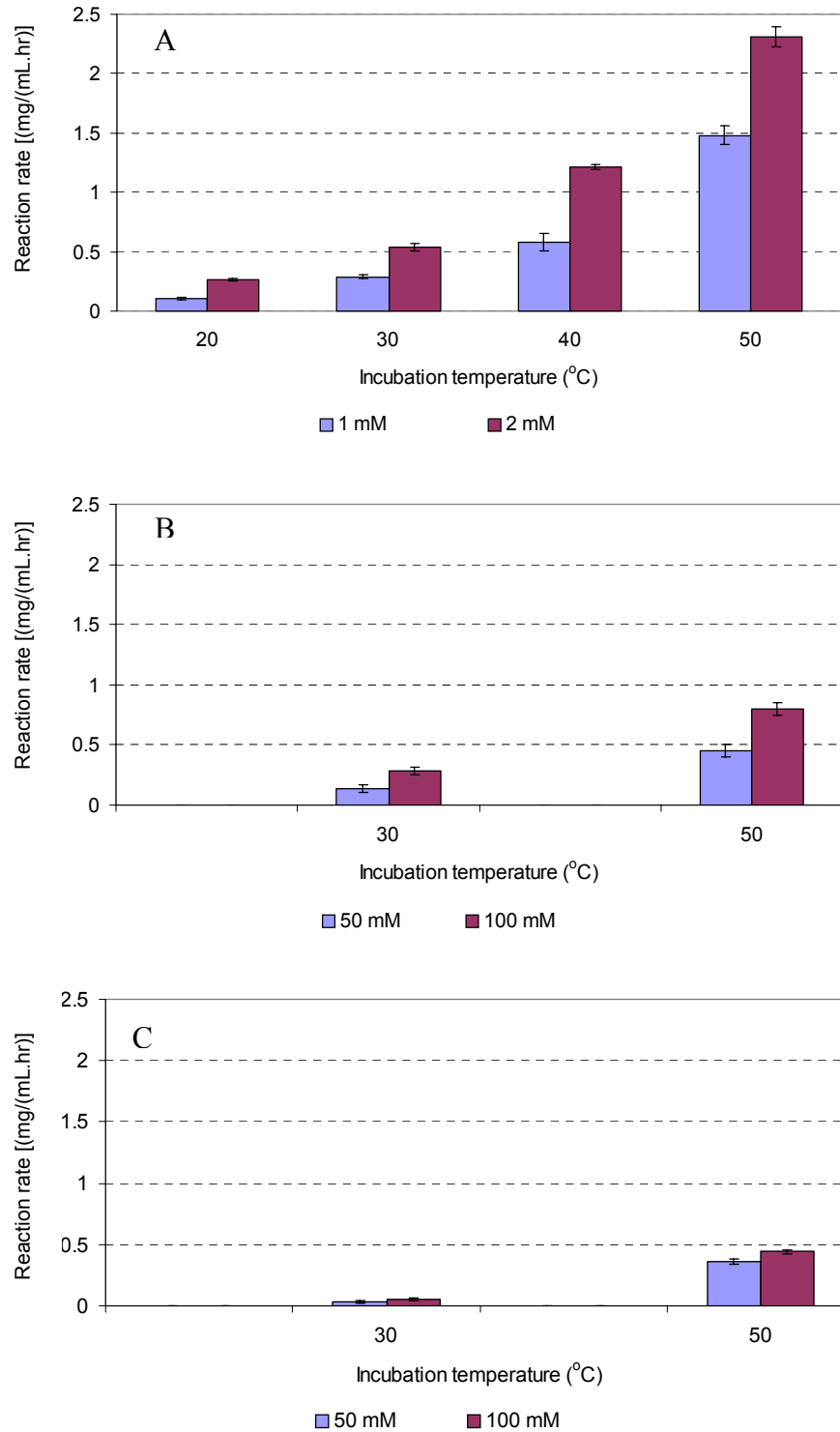


Figure 3.5: The relative reaction rates in all treatments with defatted soy flour. A is the incubation of defatted soy flour with 1 and 2 mM glutaraldehyde at 20, 30, 40 and 50°C

respectively. B is the incubation of defatted soy flour with 50 and 100 mM formaldehyde at 30 and 50°C. C is the incubation of defatted soy flour with 50 and 100 mM glyceraldehyde at 30 and 50°C respectively. Values are the means of duplicate measurements. Error bars represent the standard error of the means.

consistent with previous work on these molecules (Gerrard et al. 2003b). As expected, the reaction rate increased to about 2-fold in all cases when doubling the concentration, except incubation with glyceraldehyde. At 30°C, glyceraldehyde did not increase the reaction rate. At 50°C, the reaction rates increased, but increasing concentration from 50 to 100 mM did not increase reaction rate significantly.

3.2.2 Glycinin

SDS-PAGE: In order to establish whether the reactivity of individual subunits was influenced by other proteins present in the incubating mixture, the cross-linking experiments were repeated on glycinin, a fractionated soy protein (Peterson and Wolf 1988). Figures 3.6, 3.7 and 3.8 represent typical results obtained for the reaction of glycinin with glutaraldehyde, formaldehyde and glyceraldehyde, respectively.

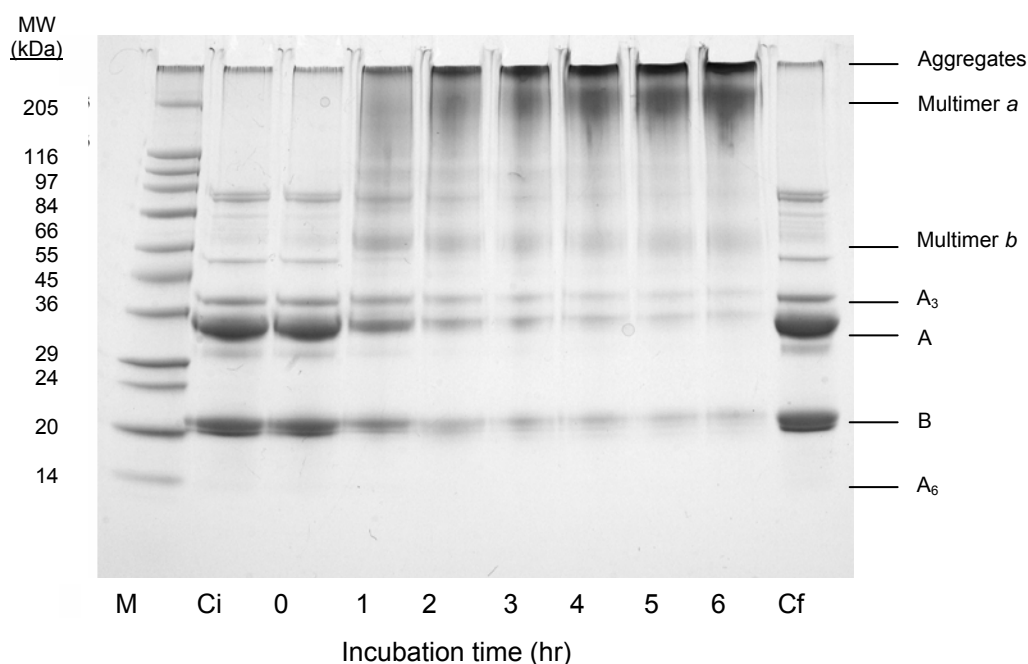


Figure 3.6: A typical SDS-PAGE profile of glycinin incubated with 1 mM glutaraldehyde. Incubation condition: 2 mg of glycinin was incubated with 1 mM glyceraldehyde at 30°C in 1 mL solution. M = Marker; Ci = Control at initial

incubation; Cf = Control at final incubation time. The image is a representative of duplicate gels.

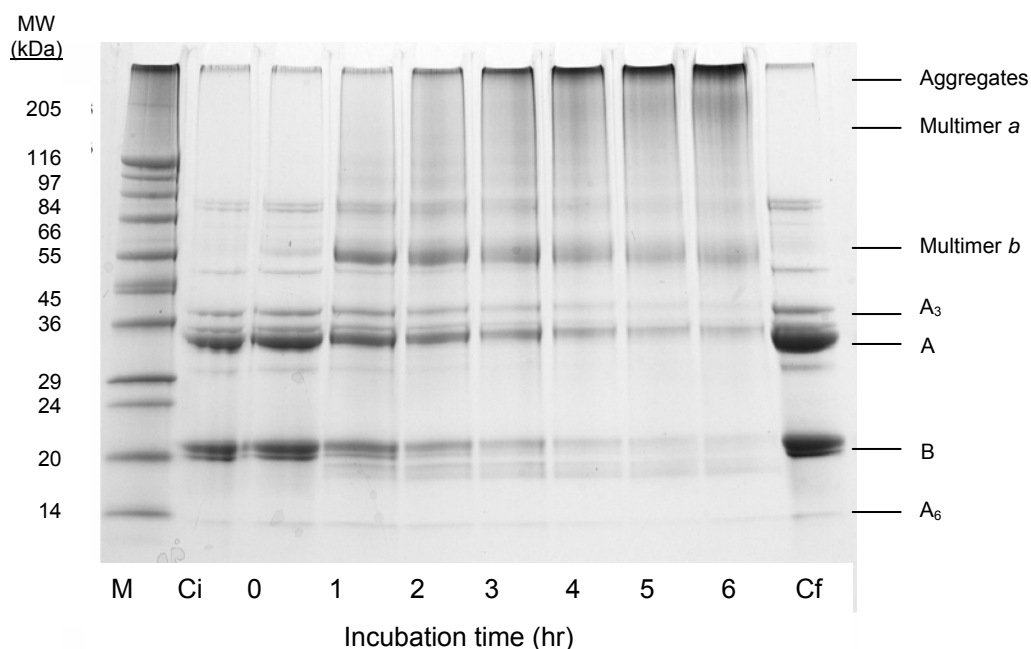


Figure 3.7: A typical SDS-PAGE profile of glycinin incubated with 50 mM formaldehyde. Incubation condition: 2 mg of glycinin was incubated with 50 mM formaldehyde at 30°C in 1 mL solution. M = Marker; Ci = Control at initial incubation; Cf = Control at final incubation time. The image is a representative of duplicate gels.

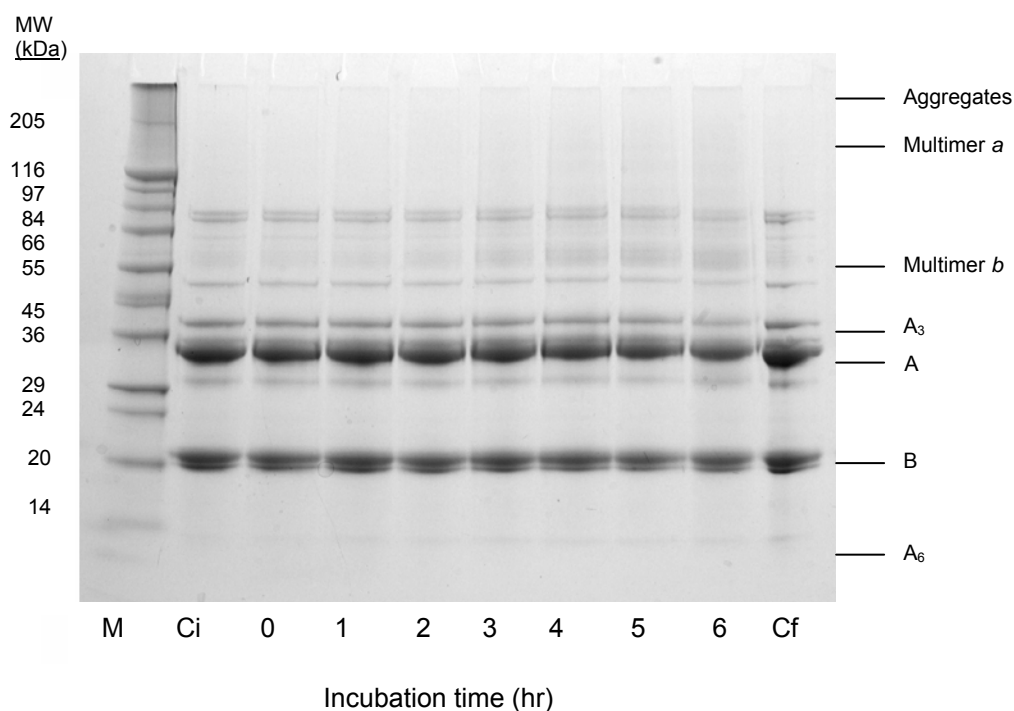


Figure 3.8: A typical SDS-PAGE profile of glycinin incubated with 50 mM glyceraldehyde. Incubation condition: 2 mg of glycinin was incubated with 50 mM glyceraldehyde at 30°C in 1 mL solution. M = Marker; Ci = Control at initial incubation; Cf = Control at final incubation time. The image is a representative of duplicate gels.

During cross-linking using glutaraldehyde (Figure 3.6), the intensities of A and B subunits progressively reduced during incubation. At the same time, the multimeric and aggregated proteins were formed and separated in three distinct regions. The aggregated proteins could not enter the gel, whilst multimers *a* and *b* are multimeric proteins around 205 and 60 kDa. These multimeric proteins were not present in the control proteins and had an unaltered intensity throughout the incubation.

When glycinin was incubated with formaldehyde (Figure 3.7), the intensities of A and B subunits also decreased, and formed aggregated proteins with increasing intensity. Multimers *a* and *b* are the same two regions of multimeric proteins as for glutaraldehyde, but the formation was at a slower rate. The intensity of multimer *b* (about 60 kDa) which was initially intense at 1 hr's incubation slowly decreased over time, unlike in the glutaraldehyde case. This suggests that multimer *b* was cross-linked to form larger aggregates.

In those experiments using defatted soy flour, the incubation with glyceraldehyde resulted in the least cross-linking. The SDS-PAGE profile (Figure 3.8) shows that the intensities of A and B subunits remained unchanged during the course of cross-linking. The formation of aggregated proteins was found to be at the slowest rate, with only a small amount of protein appearing in multimers *a* and *b*.

The appearance of aggregated proteins and multimeric proteins (multimer *a*) is consistent with those results seen for defatted soy flour (Figures 3.1, 3.2 and 3.3), but the proteins in multimer *b* were not seen in the earlier incubations, suggesting that they are specific to the cross-linked glycinin fraction.

Remaining protein: The changes in intensities on SDS-PAGE gel were quantitated by densitometry. A typical pattern of remaining proteins during incubation of glycinin with cross-linking reagents at a range of temperature fitted an exponential decay curve to obtain the first order kinetic equations as presented in Figure 3.9. Slopes

of the linear equations in this table represent the reaction rates for each treatment. A comparison of reaction rates is presented in Figure 3.10.

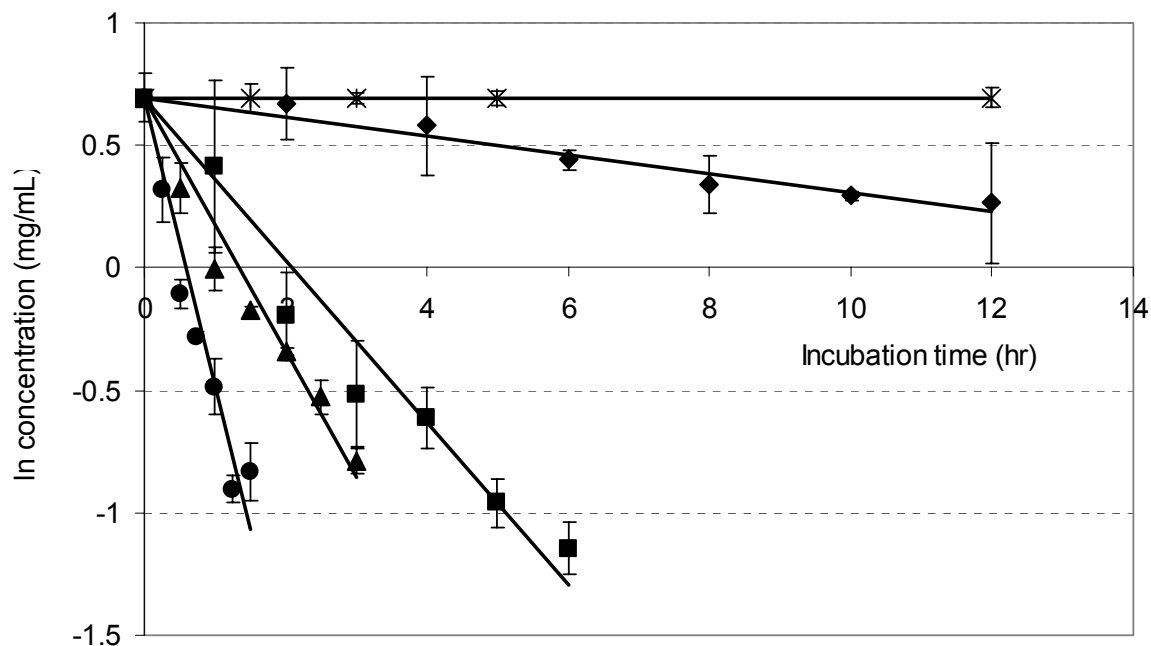


Figure 3.9: Typical patterns of linear relationships of glycine during incubation. Slopes of these lines were used as a measure of the rate of protein loss for all incubations. Values are the means of duplicate measurements. Error bars represent the standard error of the means. Key: - x = control; ◆ = 20°C; ■ = 30°C; ▲ = 40°C; ● = 50°C.

As in Figure 3.5 (defatted soy flour), the reactivity of glycine (Figure 3.10) increased by approximately two-fold with a two-fold increase in glutaraldehyde and formaldehyde concentration, except with glutaraldehyde at 20°C, in which the increment was about seven-fold. The cross-linking using glyceraldehyde did not show an increment in the reactivity, similar with those observed in the defatted soy flour. No cross-linking reactivity was observed at 30°C. At 50°C, increasing glyceraldehyde did not change the reaction rate significantly. Therefore, the cross-linking of glycine with glyceraldehyde was least reactive, as with defatted soy flour.

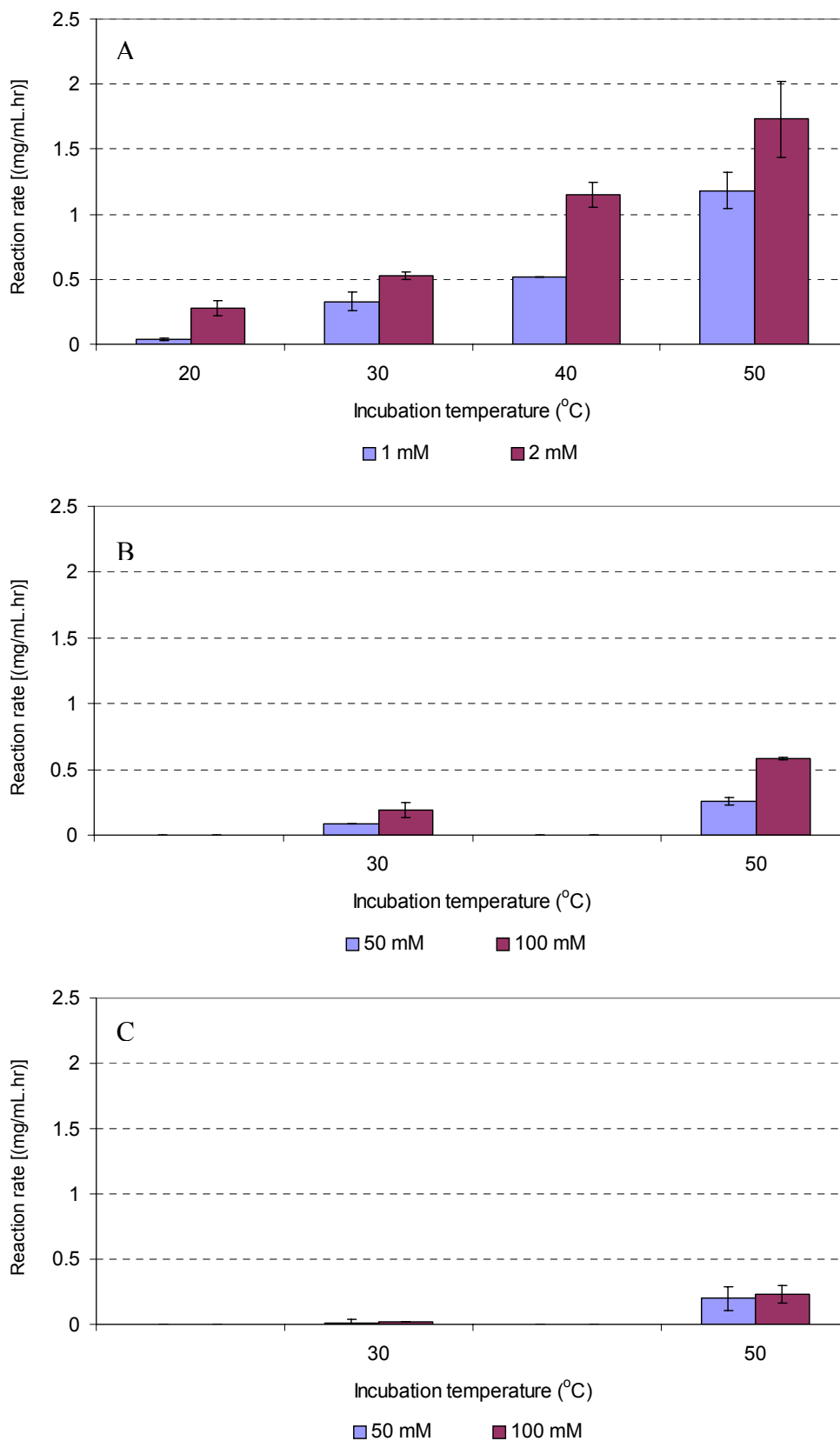


Figure 3.10: The relative reaction rates in all treatments with glycinin. A is the incubation of glycinin with 1 and 2 mM glutaraldehyde at 20, 30, 40 and 50°C respectively. B is the incubation of glycinin with 50 and 100 mM formaldehyde at 30 and 50°C. C is the incubation of glycinin with 50 and 100 mM glyceraldehyde at 30 and

50°C respectively. Values are the means of duplicate measurements. Error bars represent the standard error of the means.

3.2.3 β -Conglycinin

SDS-PAGE: β -Conglycinin was also incubated with glutaraldehyde, formaldehyde and glyceraldehyde. Typical patterns of SDS-PAGE are presented in Figures 3.11, 3.12 and 3.13.

During cross-linking using glutaraldehyde (Figure 3.11), the intensities of α' , α and β subunits progressively reduced during incubation. At the same time, the aggregated and multimeric (Multimer *a*) proteins were formed. The cross-contaminating subunits, A and B subunits of glycinin were also cross-linked.

When β -conglycinin was incubated with formaldehyde (Figure 3.12), the intensities of α' , α and β subunits also decreased, and formed aggregated and multimeric (Multimer *a*) proteins, as in the glutaraldehyde incubation, but at a slower rate.

As with those experiments with defatted soy flour or glycinin, the incubation with glyceraldehyde resulted in the least reactive cross-linking. The SDS-PAGE patterns (Figure 3.13) shows that the intensities of α' , α and β subunits remained unchanged during the course of cross-linking. The A and B subunits of glycinin were also unchanged. The formation of multimeric proteins was found to be at the slowest rate, with a faint protein appearing in multimer *a* region.

Unlike glycinin, which formed three separated regions, the formation of multimeric and cross-linked proteins appeared in one region, multimer *a*, similar to those in defatted soy flour.

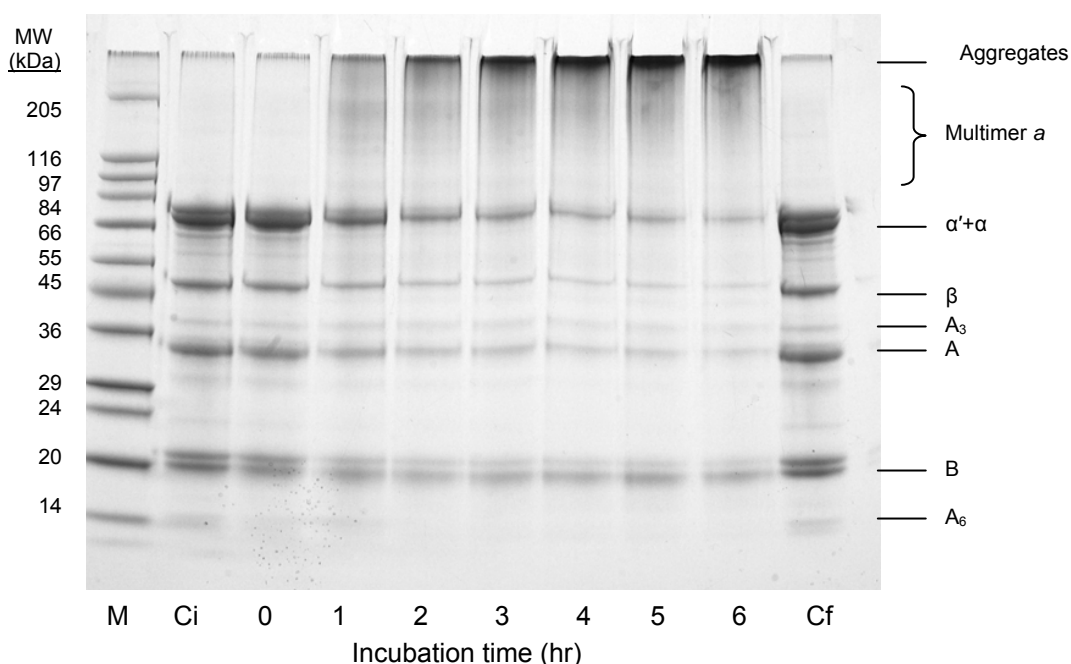


Figure 3.11: A typical SDS-PAGE profile of β -conglycinin incubated with 1 mM glutaraldehyde. Incubation condition: 2 mg of glycinin was incubated with 1 mM glyceraldehyde at 30°C in 1 mL solution. M = Marker; Ci = Control at initial incubation; Cf = Control at final incubation time. The image is a representative of duplicate gels.

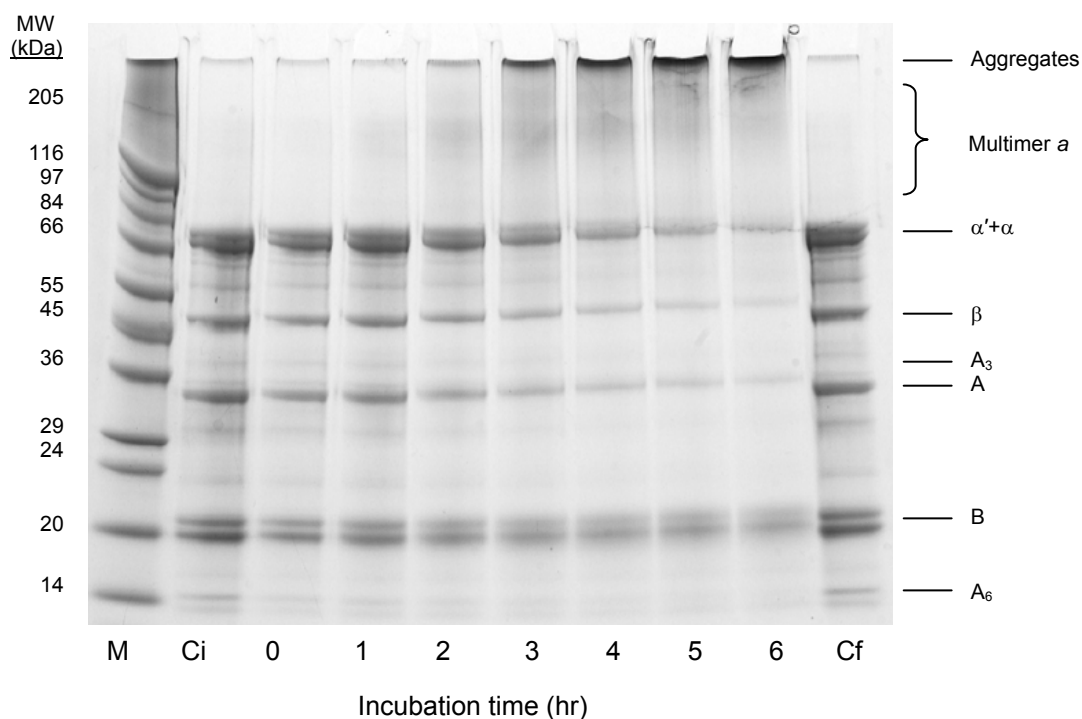


Figure 3.12: A typical SDS-PAGE profile of β -conglycinin incubated with 50 mM formaldehyde. Incubation condition: 2 mg of β -conglycinin was incubated with 50 mM formaldehyde at 30°C in 1 mL solution. M = Marker; Ci = Control at initial incubation; Cf = Control at final incubation time. The image is a representative of duplicate gels.

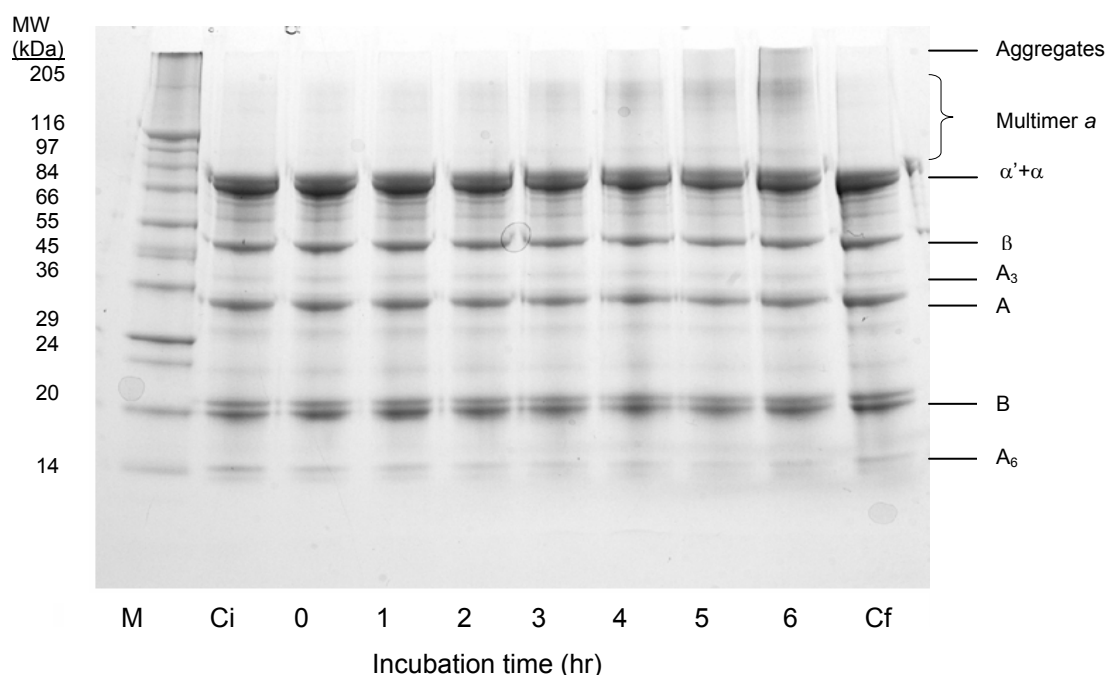


Figure 3.13: A typical SDS-PAGE profile of β -conglycinin incubated with 50 mM glyceraldehyde. Incubation condition: 2 mg of β -conglycinin was incubated with 50 mM glyceraldehyde at 30°C in 1 mL solution. M = Marker; Ci = Control at initial incubation; Cf = Control at final incubation time. The image is a representative of duplicate gels.

Remaining protein: The changes in intensities on SDS-PAGE gel were quantified by densitometry. A typical pattern of remaining protein during incubation of glycinin with cross-linking reagents at a range of temperature fitted an exponential decay curve and first-order kinetic equation (Figure 3.14). Slopes of the linear equations in this table represent the reaction rates for each treatment. A comparison of reaction rates is presented in Figure 3.15.

In comparison to Figure 3.5 (defatted soy flour) and Figure 3.10 (glycinin), in most cases, the reactivity in β -conglycinin (Figure 3.15) was increased in a range 2.3- and 4.5-fold (more than 2-fold) with two-fold increment of glutaraldehyde and formaldehyde. The reactivity in using glyceraldehyde remained slowest, similar to those results for defatted soy flour and glycinin. These results indicate that when glycinin and β -conglycinin are separated, β -conglycinin became more reactive than glycinin. This was also in agreement with an electrophoretic profile of defatted soy flour cross-linked

with glutaraldehyde (Figure 3.1). When glycinin and β -conglycinin were unfractionated (in defatted soy flour), the β -conglycinin fraction was more susceptible to cross-linking than the glycinin fraction.

The higher reactivity of β -conglycinin than glycinin was expected, since the β -conglycinin fraction had a higher hydrophilicity than glycinin, as shown by RP-HPLC chromatogram (Figure 2.8B and 2.8C Chapter 2). Lysine and arginine residues of protein are reacted with carbonyl compounds in Maillard-type cross-linking (Marquie 2001, Miller et al. 2003). Being hydrophilic amino acid residues, they are available at the surface of the protein. The accessibility of cross-linking reagents to react with these residues is more efficient than when these are buried in the protein molecule (Creighton 1993).

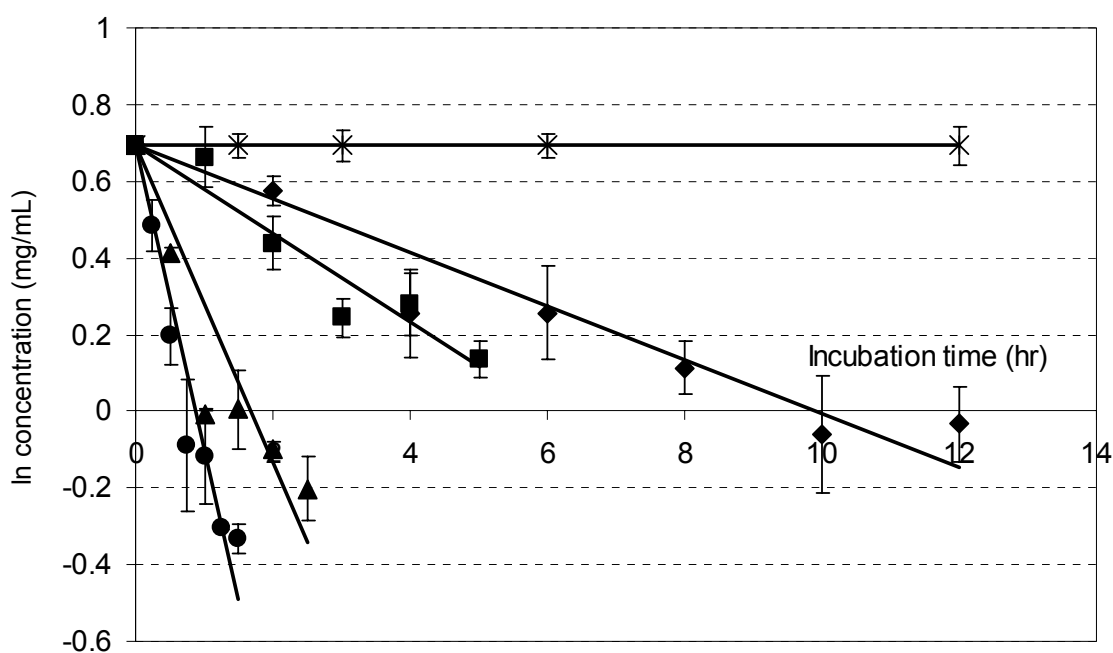


Figure 3.14: Typical patterns of linear relationships of β -glycinin during incubation. Slopes of these lines were used as a measure of rates of protein loss for all incubations. Values are the means of duplicate measurements. Error bars represent the standard error of the means. Key:- x = control; ♦ = 20°C; ■ = 30°C; ▲ = 40°C; ● = 50°C.

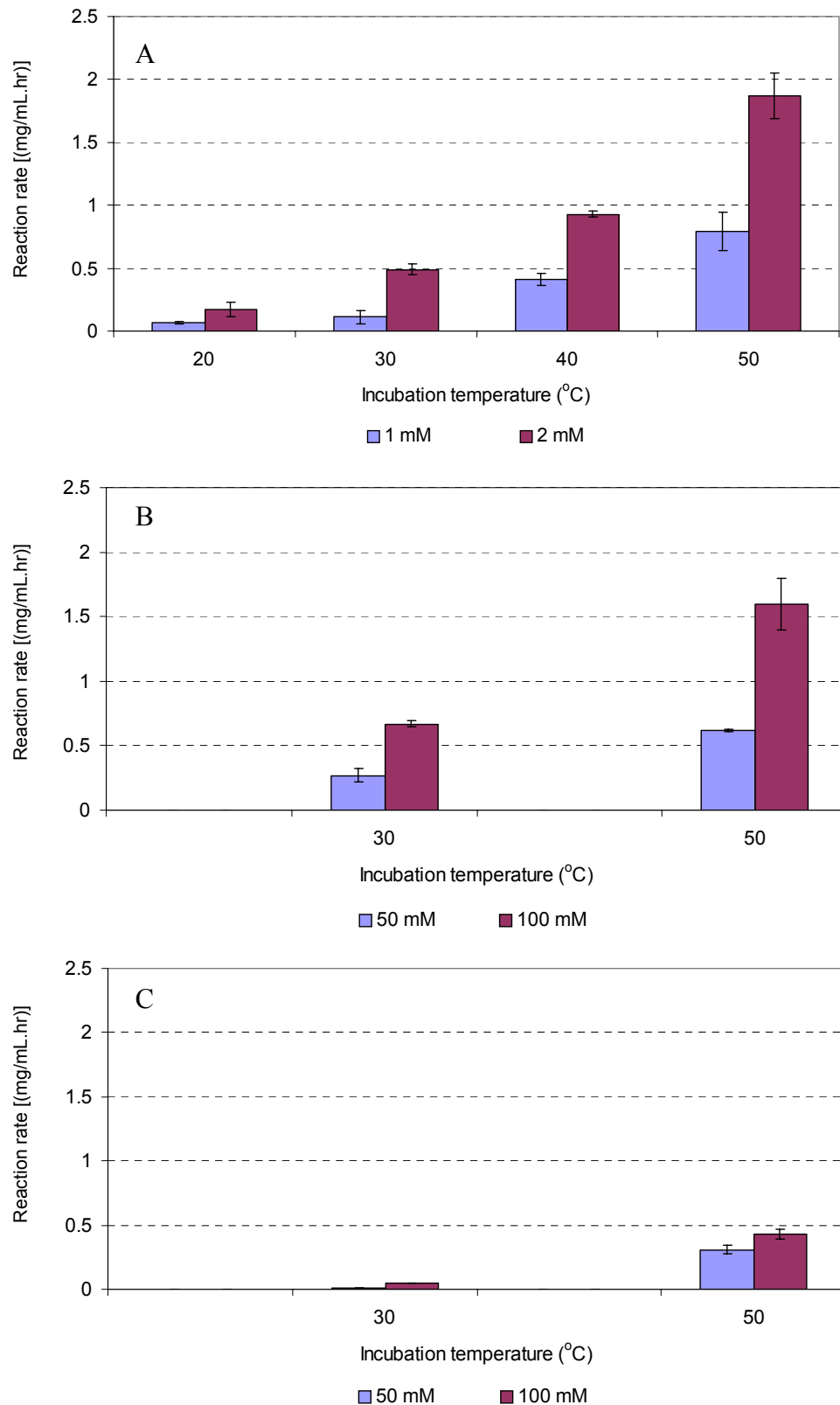


Figure 3.15: The relative reaction rates in all treatments with β -conglycinin. A is the incubation of β -conglycinin with 1 and 2 mM glutaraldehyde at 20, 30, 40 and 50°C respectively. B is the incubation of β -conglycinin with 50 and 100 mM formaldehyde at 30 and 50°C. C is the incubation of β -conglycinin with 50 and 100 mM glyceraldehyde at 30 and 50°C respectively. Values are the means of duplicate measurements. Error bars represent the standard error of the means.

3.3 SE-HPLC analysis of cross-linking reaction

In order to obtain an independent measurement of the rate of the protein cross-linking to corroborate the densitometric analysis, SE-HPLC was employed. The SE-HPLC analysis was carried out using incubation of glycinin and β -conglycinin with glutaraldehyde. The main purpose was to compare the reaction rates between SDS-PAGE and SE-HPLC techniques, under similar incubation conditions. Defatted soy flour was not used due to the precipitation during incubation that may have blocked the column.

For comparison purposes, 2 mg glycinin or β -conglycinin was incubated with 1 mM glutaraldehyde at 20°C in a 1 mL solution. The run time was fixed at 40 min. Typical patterns of SE-HPLC chromatograms of glycinin and β -conglycinin are presented in Figures 3.16 and 3.18.

3.3.1 Glycinin

Figure 3.16 shows the SE-HPLC traces of the total protein of glycinin incubated with glutaraldehyde at various points in the incubation. The chromatograms indicate a range of molecular weights centred at 66 kDa. Peaks A and B represent aggregated protein, whilst peaks C and D are the individual subunits of glycinin. During incubation, peaks A and B steadily increased, while peaks C and D were progressively decreased. The molecular size of cross-linked protein peak A was greater than 670 kDa, suggesting that it correlated with the aggregated material which could not enter the PAGE gel.

The SE-HPLC also facilitates the quantification of protein loss and formation of cross-linked proteins. Unlike SDS-PAGE, which disallowed larger molecule to enter the gel matrix, the SE-HPLC chromatogram included this material. The peak areas under the chromatogram at injection intervals were calculated for each trace to obtain the composition of protein in each 10 μ L injection volume. Plotting the amount of glycinin

subunits using a natural logarithm of the first-order kinetic equation, did not give a linear relationship (Figure 3.17). Thus the SE-HPLC results do not correlate with the

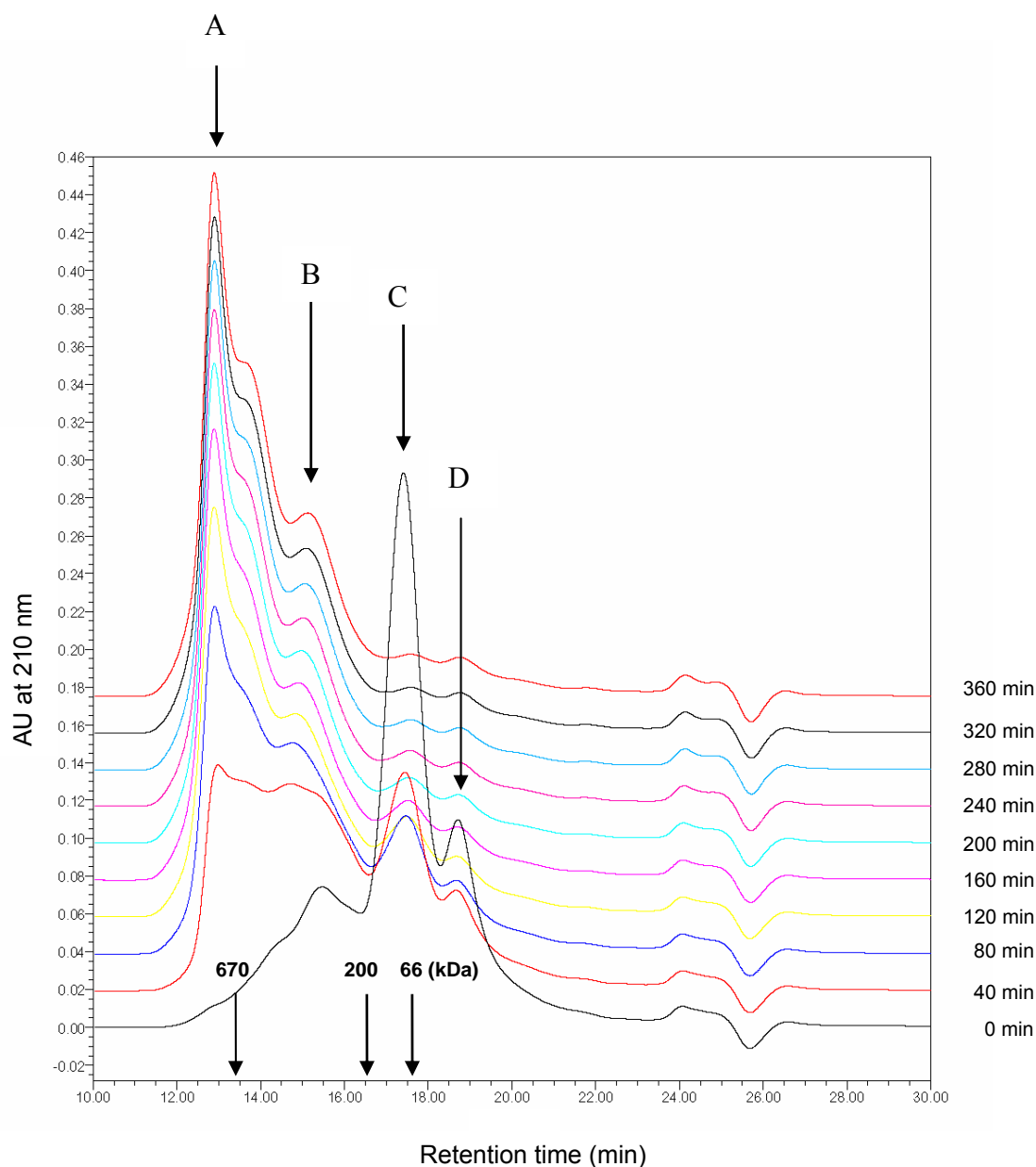


Figure 3.16: A typical series of SE-HPLC chromatograms of glycinin reacted with glutaraldehyde. Incubation conditions: 2 mg glycinin incubated with 1 mM glutaraldehyde at 20°C in 1 mL solution. 10 μ L of sample was injected onto the column. Peaks A and B are aggregated proteins. Peaks C and D are monomers of glycinin. The chromatograms are representative of duplicate experiments.

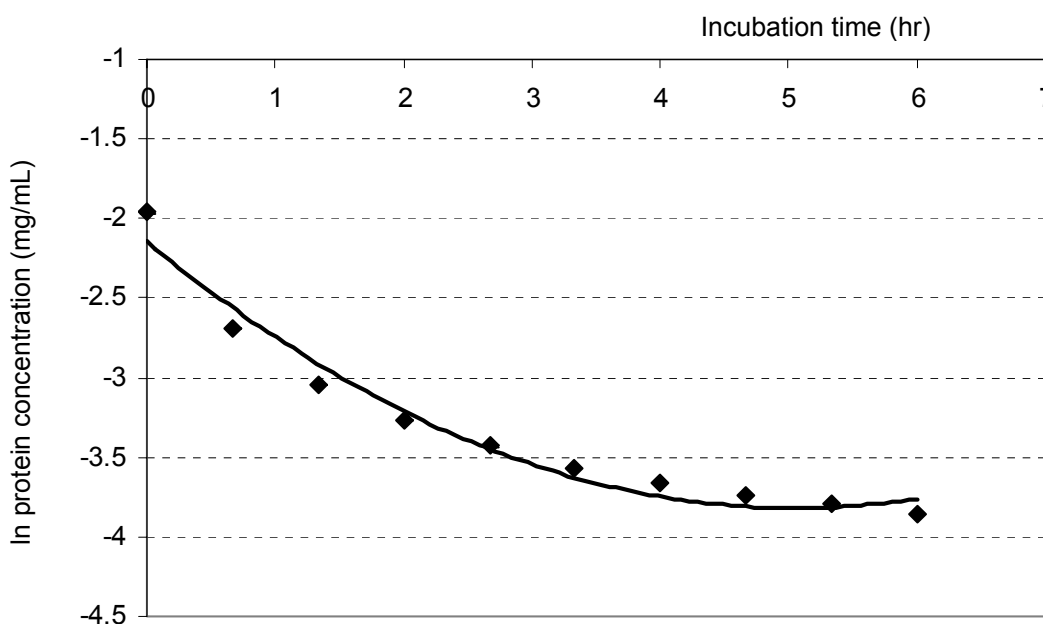


Figure 3.17: Exponential-like curve of ln protein concentration versus incubation time.

SDS-PAGE results, presumably due to the presence of large aggregates in the SE-HPLC analysis which were excluded from the SDS-PAGE gel.

3.3.2 β -Conglycinin

Figure 3.18 shows SE-HPLC traces of the total protein of β -conglycinin incubated with glutaraldehyde at various points in the incubation. The chromatograms indicate a range of molecular weights centred at 66 kDa. Peaks A* and B* represent aggregated protein, whilst peaks C* to J* are the protein subunits in a range of molecular sizes.

Individual peak areas under the chromatograms were quantitated. Based on the subunit profiles, there were three groups of reactivity in β -conglycinin. Group 1 consists of the cross-linked proteins (peaks A* and B*), in which the protein amount increased during incubation. Group 2 consists β -conglycinin subunits (peaks C*, D* and E*), in which the protein amount reduced over time. Group 3 consists of the protein subunits (peaks F*, G*, H*, I* and J*) that were not cross-linked during incubation.

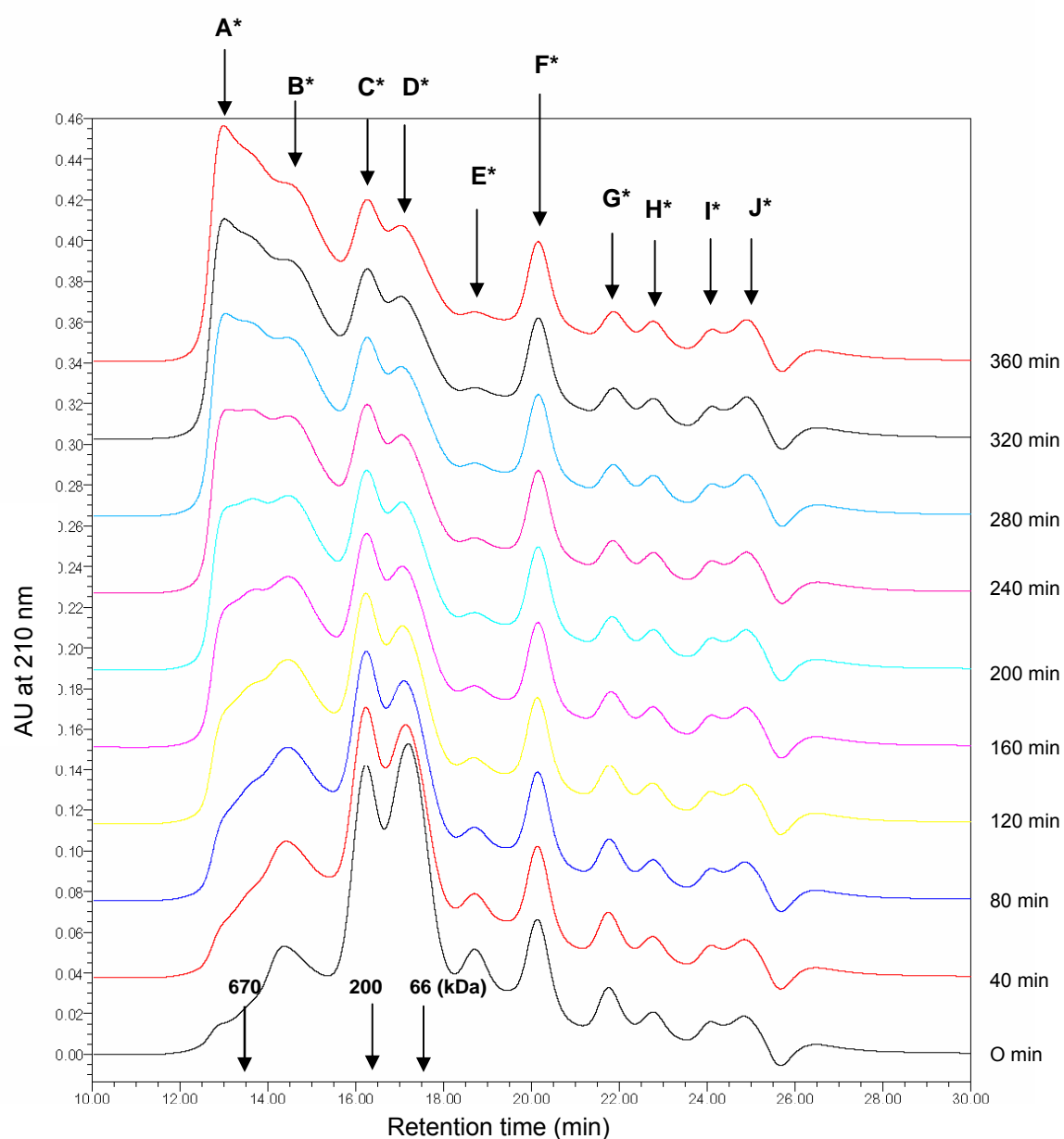


Figure 3.18: A typical series of SE-HPLC chromatograms from of β -conglycinin with glutaraldehyde. Incubation condition: 1 mg glycinin incubated with 1 mM glutaraldehyde at 20°C in 500 μ L solution. 10 μ L of sample was injected onto the column. Injection interval was set-up at 40 min. Peaks A* and B* are aggregated proteins. Peaks C*-J* are monomers of β -conglycinin. These chromatograms are representative of duplicate experiments.

For kinetic analysis, the loss of protein subunits (peaks C*, D* and E*) was quantitated. When this was fitted with a first-order kinetic equation, a strong linear relationship against incubation time was obtained, as shown in Figure 3.19, suggesting that the loss of protein subunits in β -conglycinin followed first-order kinetic reaction. The reaction rate, obtained from the slope of the equation was compared with that obtained from densitometric analysis (Figure 3.15A) and presented in Figure 3.20. The result shows that the reaction rate obtained from SE-HPLC was about 1.5-fold higher than the rate from the densitometric analysis. The reduction of individual proteins (SE-HPLC) appeared to be cross-linked faster when measured by SE-HPLC rather than SDS-PAGE although the trends were similar. In later experiments, only SDS-PAGE technique was employed, as this technique allowed a more comprehensive analysis of the impact on individual proteins in the soy flour.

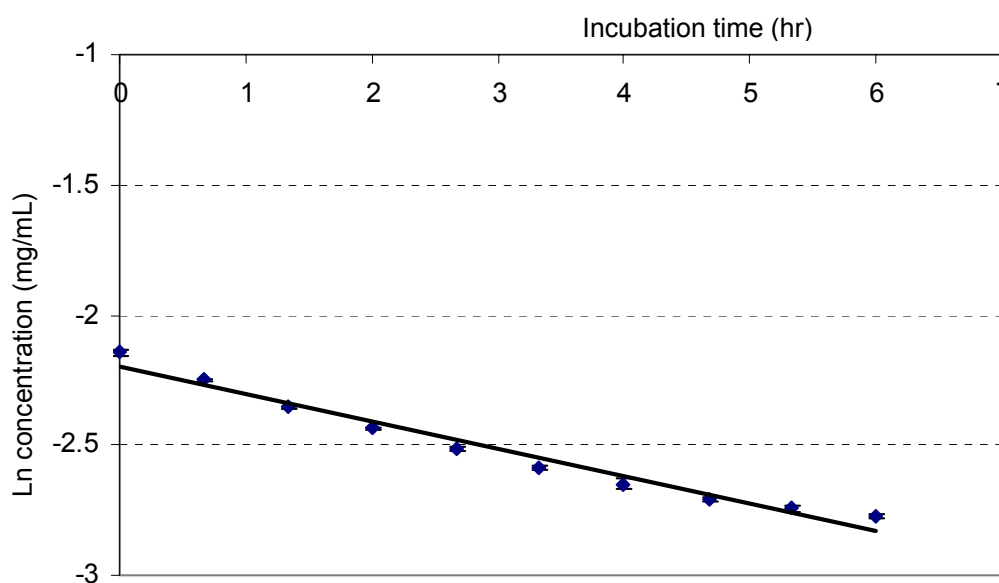


Figure 3.19: A linear relationship between the lost of protein subunits of β -conglycinin during incubation. The slope of this line was used as a measure of rate of protein loss during incubation. Values are the means of duplicate measurements. Error bars represent the standard error of the means.

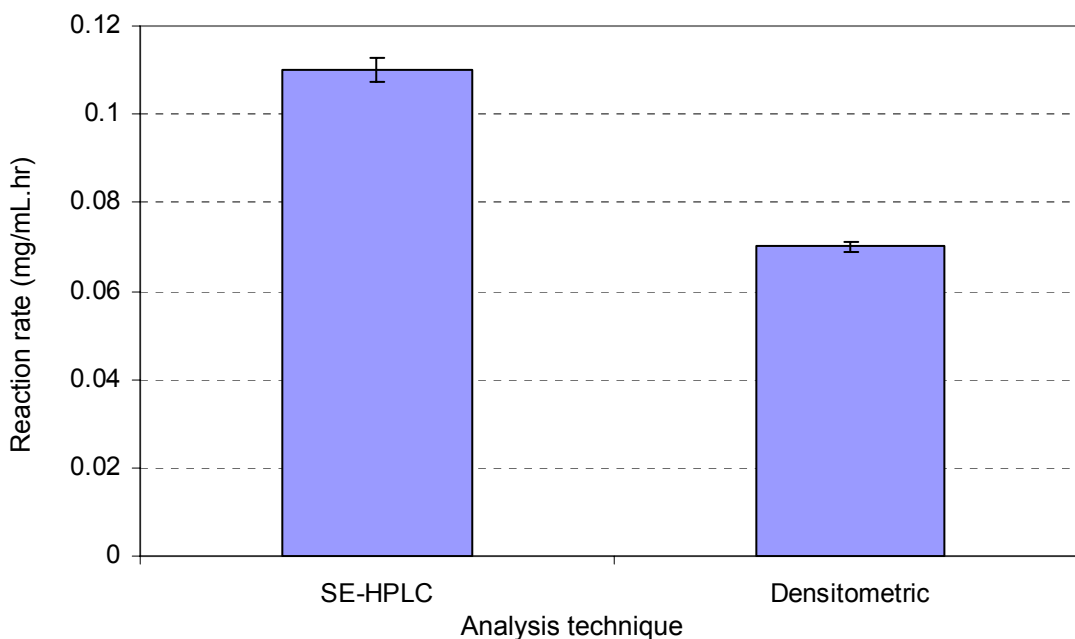


Figure 3.20: Relative reaction rates from SE-HPLC and densitometric analysis techniques under the same incubation condition (Using 1 mM glutaraldehyde at 20°C). Values are the means of duplicate measurements. Error bars represent the standard error of the means.

3.4 Relative rates of cross-linking of individual proteins

Interesting results were found on the reaction rates in defatted soy flour, which contained unfractionated glycinin and β -conglycinin. In this case, the incubation of defatted soy flour with glutaraldehyde is used as an example to show various reaction rates of protein subunits in a range of temperatures and glutaraldehyde concentrations, as presented in Figure 3.21.

In this unfractionated glycinin and β -conglycinin (Figure 3.21) (Peterson and Wolf 1988), the A subunits of glycinin had the highest reaction rate, followed by $\alpha'+\alpha$ and β subunits of β -conglycinin. B subunits were the least reactive. However, when using fractionated glycinin, the reaction rates of B reached the reaction rates of A subunits. As judged by SDS-PAGE and densitometric analysis, the reaction rates of A and B subunits are presented in Figure 3.22. This phenomenon is expected as the

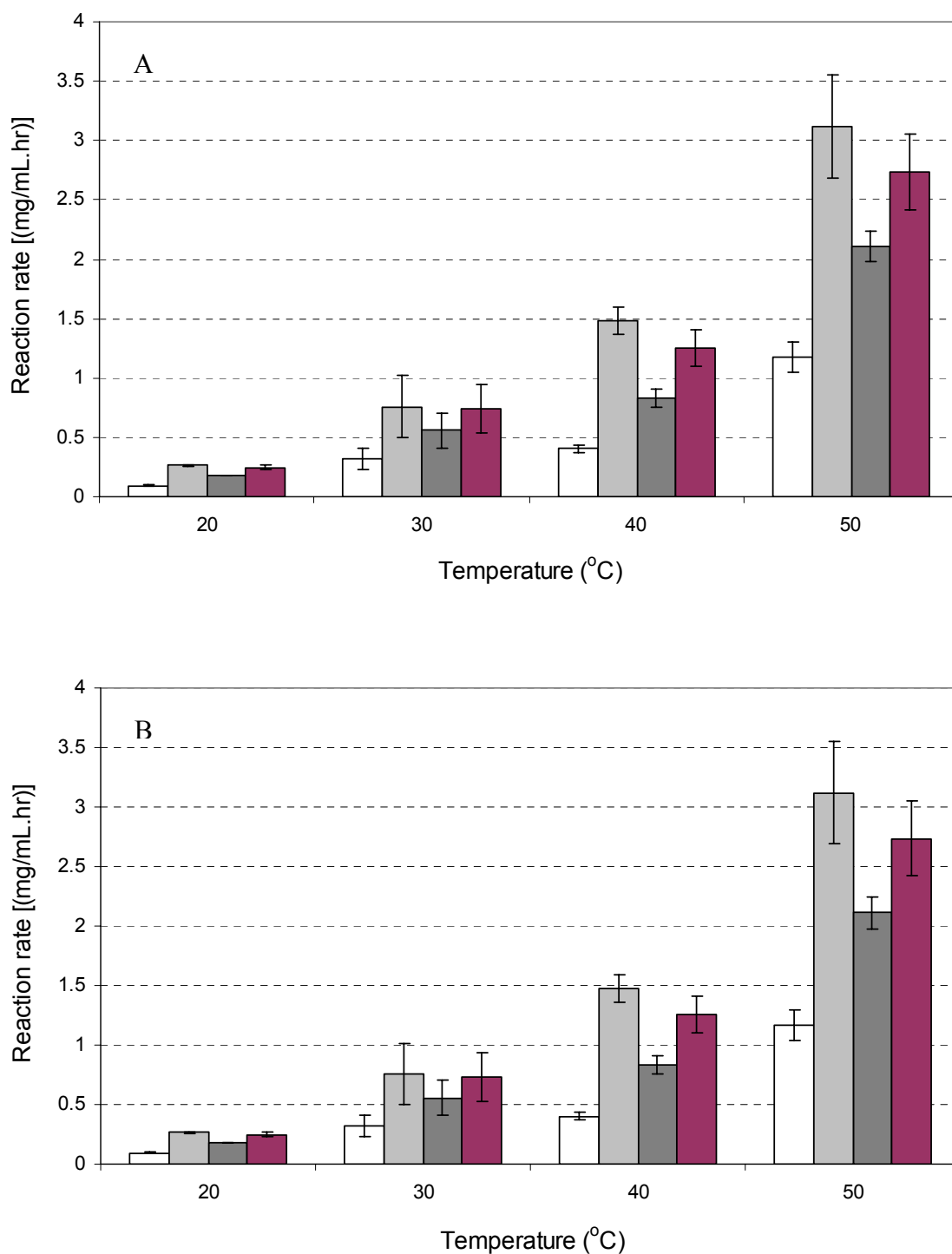


Figure 3.21: The reaction rate of individual subunits at a range of temperatures for the glutaraldehyde experiment. A and B are representing defatted soy flour incubated with 1 and 2 mM glutaraldehyde respectively. The values are the means of duplicate measurements. Error bars represent the standard error of the means.

Key:- □ Basic subunits, ■ Acidic subunits, ■ β subunit, ■ α'+α subunits

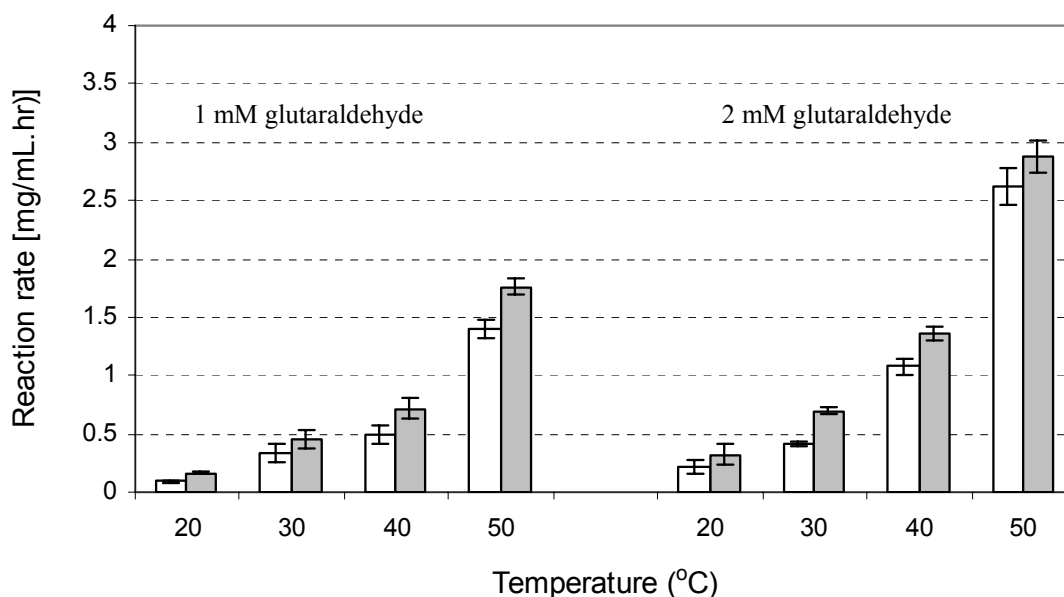


Figure 3.22: Reactive rates of glycinin incubated with glutaraldehyde at a range of concentrations and temperatures. The values are the means of duplicate measurements. Error bars represent the standard error of the means.
Key:- □ Basic subunits, ■ Acidic subunits.

disulfide bonds that linked A and B subunits were cleaved by 2-mercapethanol during extraction. These changes destabilise the conformational structure of glycinin. The amine groups of lysine and arginine residues then had increased accessibility to cross-link with glutaraldehyde, which resulted in more rapid cross-linking.

Table 3.3 shows the composition of arginine, lysine and cysteine residues in the glycinin and β -conglycinin fraction in various cultivars. Comparing both fractions, there was no general difference in arginine composition. The composition of lysine was variable according to cultivar. However, the amount of cysteine in glycinin fraction was significantly higher than that of β -conglycinin, suggesting that glycinin had more disulfide bonds. Thus, it is not surprising that the relative reactivity of the glycinin fraction increased upon fractionation.

Table 3.3: Amino acid composition in a range of soybean varieties.

Amino acid	β -Conglycinin fraction (%)				
	K1430	Hutcheson	93-90-29	KS4997-98	Range
Lysine	5.6	6.8	5.1	4.6	4.6-6.8
Arginine	7.6	6.7	9.1	6.1	6.1-9.1
Cysteine	0.6	0.5	0.5	0.5	~0.5
Amino acid	Glycinin fraction (%)				
	K1430	Hutcheson	93-90-29	KS4997-98	Range
Lysine	5.4	4.4	5.8	3.7	3.7-5.8
Arginine	6.9	9.3	7.9	6.1	6.1-9.3
Cysteine	1.0	1.1	1.1	0.7	0.7-1.1

Values represent the percentage of amino acid in 1 g protein fraction. K1430, Hutcheson, K93-90-23 and KS4997-98 are the soybean varieties. Data adapted from Khatib et al. (2002).

3.5 Conclusion

SDS-PAGE analysis clearly showed that fractionated and unfractionated soy proteins were cross-linked with glutaraldehyde, formaldehyde and glyceraldehyde and can be used to assess the reactivity of these carbonyl compounds. The reactivity evaluated from the densitometric analysis gives quantitative measurement for kinetic analysis. The reactivity of the protein observed *in vitro* need to be translated into the actual food processing system, in order to observe *in situ* consequences in tofu system. The fact that glycinin is more reactive when fractionated than in defatted soy flour is hard to explain since the amount of lysine and arginine is unchanged. However, it is likely that these residues are made more available for Maillard chemistry by the extraction process. This cross-linking chemistry will be employed in tofu (Chapter 6).

3.6 References

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CHAPTER 4

CROSS-LINKING OF SOY PROTEINS BY TRANSGLUTAMINASE

4.1 Background

In this chapter, transglutaminase (TGA) was employed to catalyse cross-linking in defatted soy flour, glycinin and β -conglycinin in an analogous fashion to the Maillard cross-linking in Chapter 3. For the TGA cross-linking, preliminary trials were undertaken and showed that the rate of cross-linking was very rapid at elevated temperatures, and not suitable for kinetic studies. Therefore, the most suitable temperature for monitoring the cross-linking reaction was at 20°C and at pH 7.0. The experimental design for this study is presented in Table 4.1. Two concentrations of TGA (500 and 1000 ppm) were employed, based on their impacts on the subunit changes detected on SDS-PAGE. A concentration lower than 500 ppm resulted in very slow changes of subunits, whilst at a higher concentration, > 1000 ppm resulted in instant disappearance of protein subunits, and the quantification of protein loss was difficult to achieve.

As in Chapter 3, the main objective of this experiment was to determine the reaction rate of cross-linking reaction using SDS-PAGE and densitometry. The reaction rates from varying the TGA concentrations with glycinin, β -conglycinin and defatted soy flour are compared.

4.2 SDS-PAGE analysis

SDS-PAGE was run under reducing conditions and then then analysed densitometrically, as in Chapter 3. Initially, a series of TGA concentrations, 250, 500, 1000 and 2000 ppm were trialed using defatted soy flour, in order to choose the

appropriate concentrations of TGA for kinetic analysis. Preliminary results showed that the 250 ppm TGA resulted in a negligible reactivity, as shown in Figure 4.1, whilst the 2000 ppm resulted in an instant cross-linking, and most of the subunits were completely cross-linked throughout incubation, as presented in Figure 4.2. Thus, 250 and 2000 ppm were unable to be used for kinetic analysis based on the protein loss. The most suitable concentrations to quantitate the protein loss for kinetic analysis were at 500 and 1000 ppm, as shown in Figures 4.3 and 4.4.

Table 4.1: The experimental design for TGA cross-linking in defatted soy flour glycinin and β -conglycinin.

Protein source	Enzyme Concentration	Temp (°C)	Incubation period (hr)	Sampling interval (hr)
Defatted soy flour	500 ppm TGA	20	1.5	0.25
	1000 ppm TGA	20	1.5	0.25
Glycinin	500 ppm TGA	20	1.5	0.25
	1000 ppm TGA	20	1.5	0.25
β -Conglycinin	500 ppm TGA	20	1.5	0.25
	1000 ppm TGA	20	1.5	0.25

All experiments were carried out in duplicate.

4.2.1 Defatted soy flour

4.2.1.1 SDS-PAGE

SDS-PAGE profiles of defatted soy flour incubated with 500 and 1000 ppm TGA are presented in Figures 4.3 and 4.4. By using 500 ppm TGA (Figure 4.3), cross-links between soy proteins were seen to form as indicated by the gradual increase in intensity of the aggregated protein smeared on the top of the gel. At the same time, the intensity of α' , α , β , A_3 and other A subunits were seen to decrease steadily due to cross-linking.

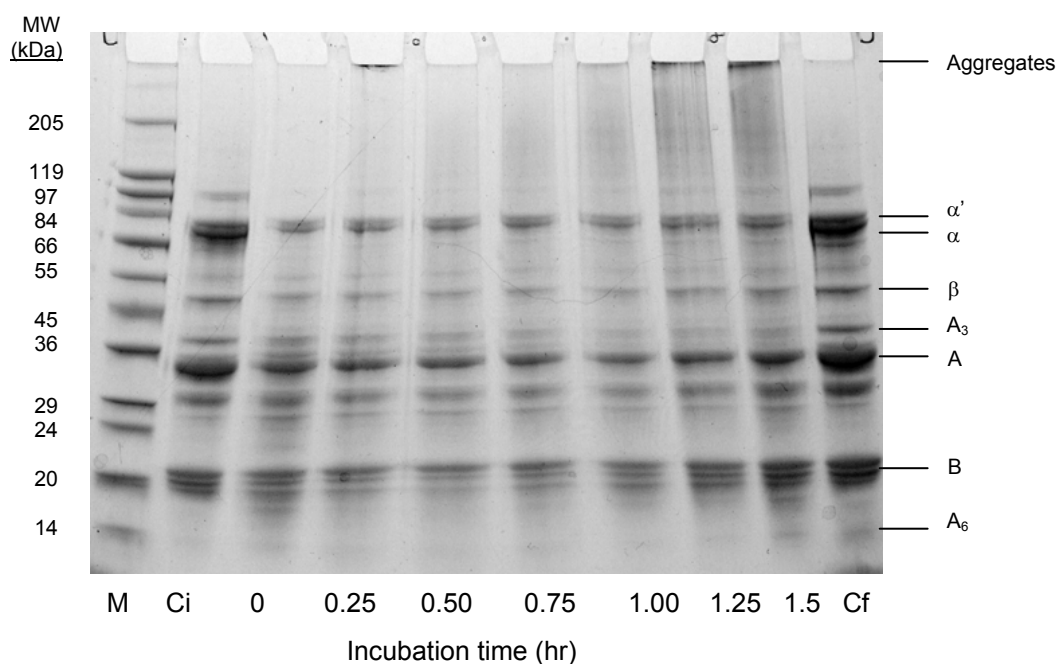


Figure 4.1: A typical SDS-PAGE profile of defatted soy flour incubated with 250 ppm TGA; M = Marker; Ci = Control initial; Cf = Control final; Incubation conditions: 6 mg of defatted soy flour were dissolved in 1 mL water and incubated with 250 ppm TGA at 20°C. The image is a representative of duplicate gels.

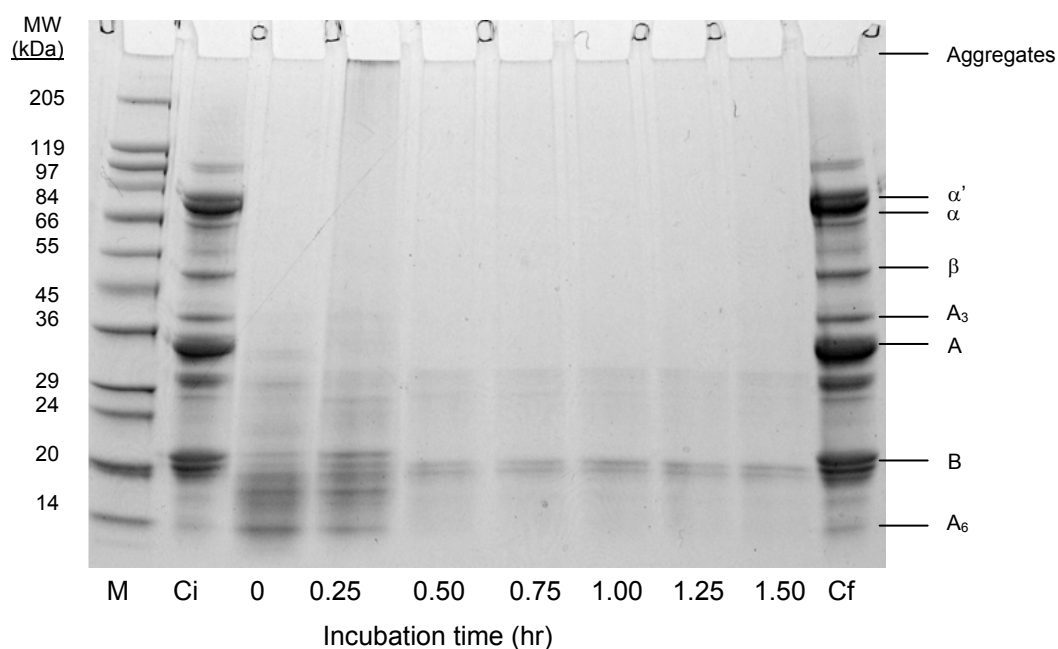


Figure 4.2: A typical SDS-PAGE profile of defatted soy flour incubated with 2000 ppm TGA; M = Marker; Ci = Control initial; Cf = Control final; Incubation conditions: 6 mg of defatted soy flour were dissolved in 1 mL water and incubated with 2000 ppm TGA at 20°C. The image is a representative of duplicate gels.

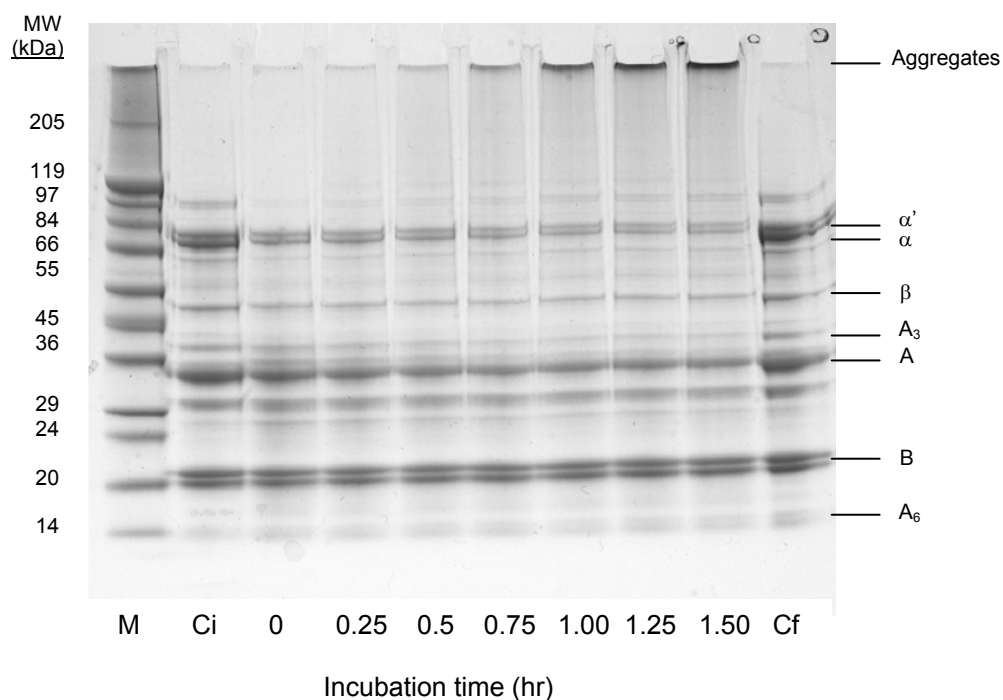


Figure 4.3: A typical SDS-PAGE profile of defatted soy flour incubated with 500 ppm TGA; M = Marker; Ci = Control initial; Cf = Control final; Incubation conditions: 6 mg of defatted soy flour were dissolved in 1 mL water and incubated with 500 ppm TGA at 20°C. The image is a representative of duplicate gels.

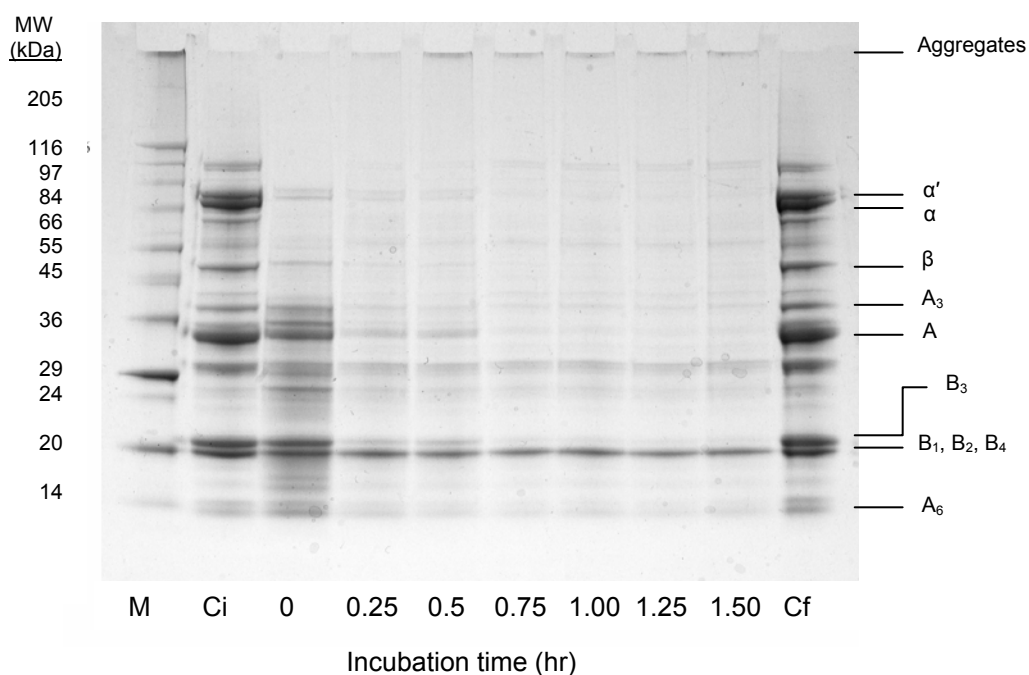


Figure 4.4: A typical SDS-PAGE profile of defatted soy flour incubated with 1000 ppm TGA; M = Marker; Ci = Control initial; Cf = Control final; Incubation conditions: 6 mg of defatted soy flour were dissolved in 1 mL water and incubated with 1000 ppm TGA at 20°C. The image is a representative of duplicate gels.

When 1000 ppm TGA was employed, the intensity of α' , α and β subunits of β -conglycinin dropped rapidly at 0 hr incubation (Figure 4.4). The intensities of A subunits were also slightly reduced. After 15 minutes incubation, the intensities of the A subunits were further reduced, followed by the B subunits. This profile showed that α' , α and β subunits were most susceptible to cross-linking by TGA.

However, within the B subunits, the B₃ polypeptide was cross-linked, but the B₁, B₂ and B₄ polypeptides remained stable throughout incubation. This phenomenon was in agreement with the electrophoretic results of earlier investigations using soy proteins (Basman et al. 2002, Ikura et al. 1980, Kang et al. 1994, Tang et al. 2006, Zhang et al. 2003). Their differences in reactivities are presumably due to the substrate specificity of TGA (Grootjans et al. 1995, Han and Damodaran 1996, Kamiya et al. 2003, Shimba et al. 2002, Taguchi et al. 2000)

4.2.1.2 Remaining proteins

The changes of intensities from Figures 4.3 and 4.4 were analysed densitometrically to quantitate the remaining protein in the solution over incubation time. In contrast to the cross-linking using carbonyl compounds, where unreacted protein concentration at $t=0$ was equal to control initial (C_i) (Chapter 3), the unreacted protein concentration at $t=0$ in TGA cross-linking decreased instantly, as shown in Figure 4.5. At 500 and 1000 ppm TGA, 10% and 27% of proteins were immediately cross-linked.

The concentration of unreacted protein for all treatments followed an exponential decline. These data were fitted to the natural logarithm of a first-order kinetic equation to assess the reaction rates. Typical linear fits are presented in Figure 4.6. As the concentration increased from 500 to 1000 ppm, the slope, which represents reaction rate, increased as summarised in Figure 4.14.

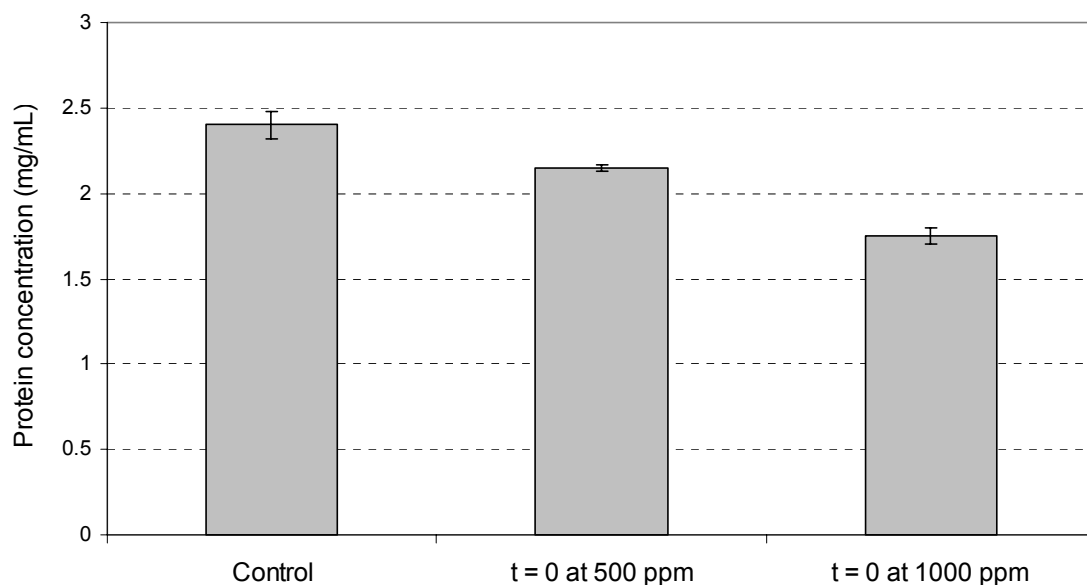


Figure 4.5: Protein concentration in defatted soy flour at t=0 of 500 and 1000 ppm TGA. Values are representatives of duplicate measurements. Error bars represent the standard error of the means.

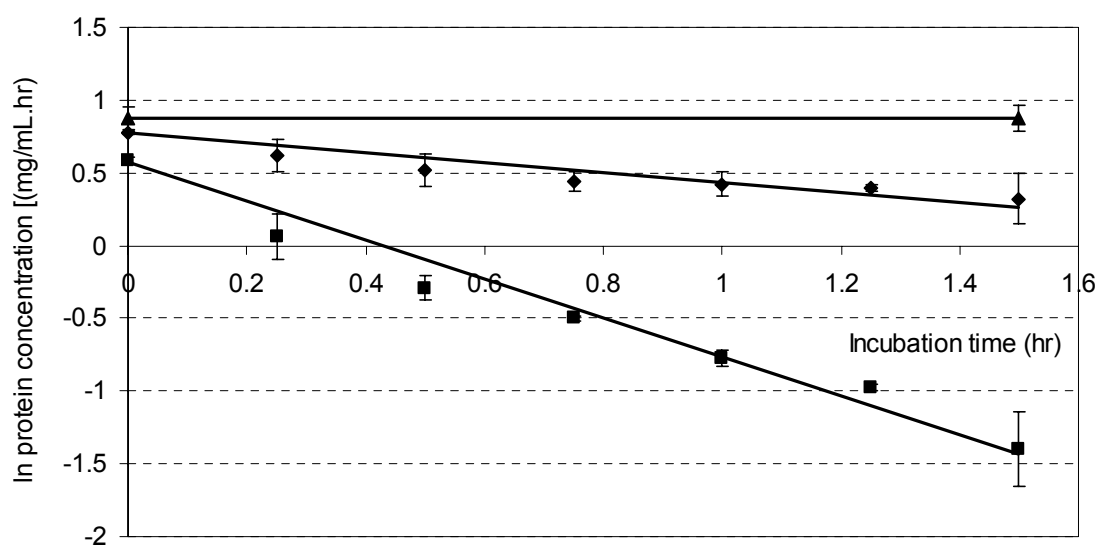


Figure 4.6: Typical linear fits of remaining protein concentration during incubation of defatted soy flour with TGA at 20°C. Values are representatives of duplicate measurements. Error bars represent the standard error of the means. ◆ = 500 ppm TGA, ■ = 1000 ppm TGA, ▲ = Control.

4.2.2 Glycinin

4.2.2.1 SDS-PAGE

SDS-PAGE profiles of glycinin incubated with 500 and 1000 ppm TGA are presented in Figures 4.7 and 4.8. Both experiments had a 15 min lag phase prior to complete cross-linking. After 15 min incubation, a rapid cross-linking of A and B subunits was observed. In the 1000 ppm incubation (Figure 4.8), A and B subunits were completely cross-linked after 30 min incubation compared to 500 ppm TGA which showed faint bands of A and B subunits (Figure 4.7). This result suggests that the A and B subunits become more accessible to higher concentration of TGA.

A rapid cross-linking of A and B subunits also indicates that these subunits were cleaved by reducing agent, 2-mercaptoethanol, during extraction and unfolded (Figure 2.2). Upon reducing the disulfide bonds, the protein denatured and the substrates in A and B became more accessible for TGA cross-linking than was observed in defatted soy flour. This is analogous to the behaviour observed for Maillard cross-linking in Chapter 3.

Although the A and B subunits showed a rapid cross-linking, the formation of cross-linked proteins was not observed on the top of the gel, due to the formation of large aggregates that could not enter the gel. These electrophoretic profiles were unable to be used for kinetic analysis of cross-linking reaction, since the sudden drop in concentration precluded the use of the standard curve fit.

4.2.2.2 Remaining proteins

Protein concentrations at $t=0$ for 500 and 1000 ppm TGA are presented in Figure 4.9. The amounts of protein lost were accounted for 18% and 30% at 500 and 1000 ppm TGA, respectively. As expected, the higher concentration resulted in most rapid cross-linking. After 1 hr incubation, 90% (1.8 mg/mL.hr) and 100% (2 mg/mL.hr) glycinin was cross-linked using 500 and 1000 ppm TGA, respectively.

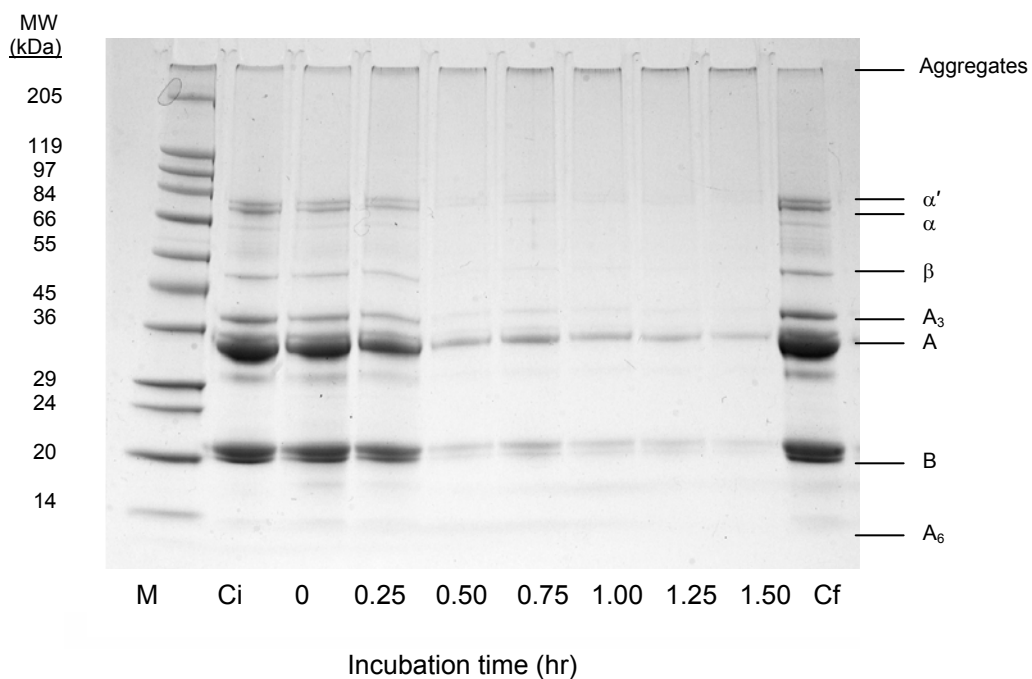


Figure 4.7: A typical SDS-PAGE profile of glycinin incubated with 500 ppm TGA; M = Marker; Ci = Control initial; Cf = Control final; Incubation condition: 2 mg glycinin was dissolved in 1 mL water and incubated with 500 ppm TGA at 20°C. The image is a representative of duplicate gels.

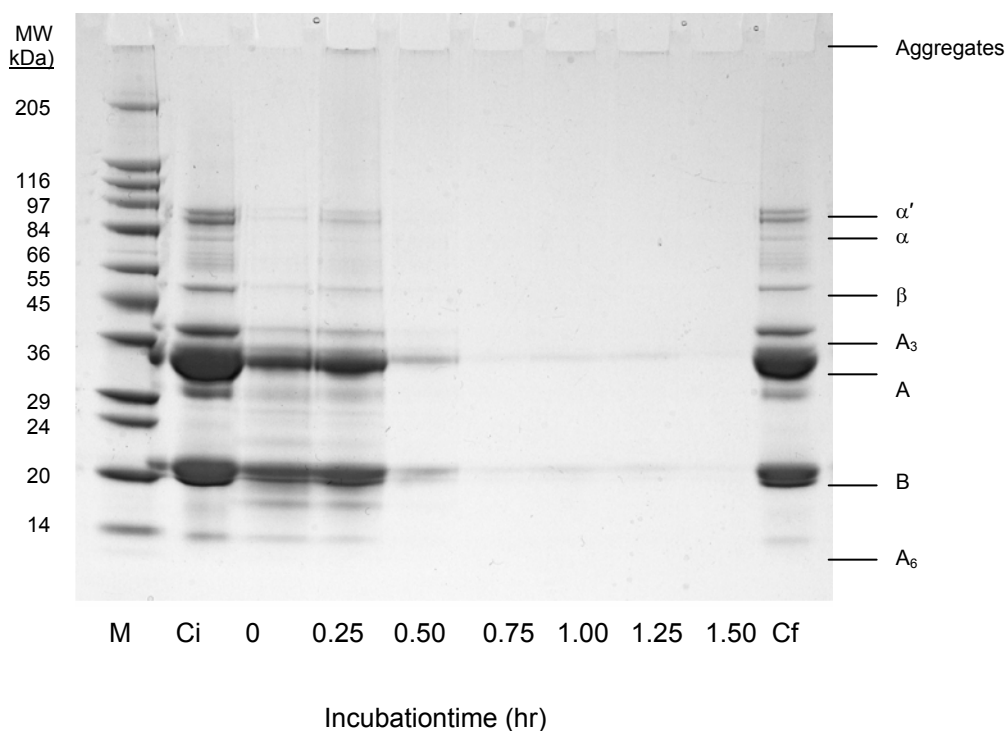


Figure 4.8: A typical SDS-PAGE profile of glycinin incubated with 1000 ppm TGA; M = Marker; Ci = Control initial; Cf = Control final; Incubation condition: 2 mg glycinin was dissolved in 1 mL water and incubated with 1000 ppm TGA at 20°C. The image is a representative of duplicate gels.

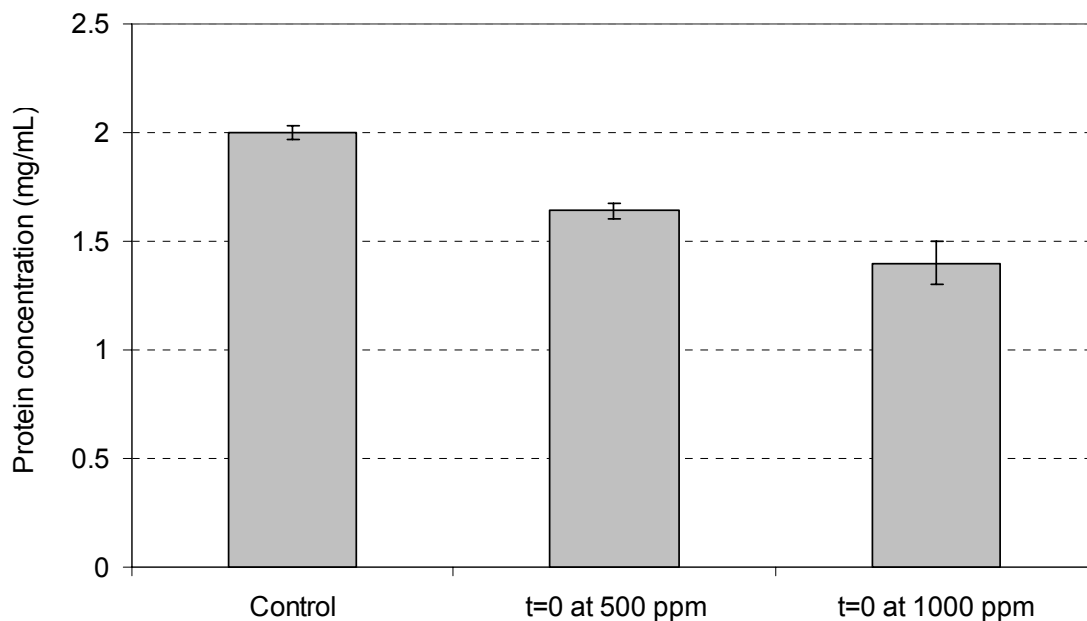


Figure 4.9: Protein concentration in glycinin at t=0 of 500 and 1000 ppm TGA. Values are representatives of duplicate measurements. Error bars represent the standard error of the means.

4.2.3 β -Conglycinin

4.2.3.1 SDS-PAGE

SDS-PAGE profiles of β -conglycinin incubated with 500 and 1000 ppm TGA are presented in Figures 4.10 and 4.11. Using 500 ppm TGA (Figure 4.10), the intensities of α' , α and β subunits gradually reduced, and concomitantly, the intensities of cross-linked proteins increased. However, at 1000 ppm TGA (Figure 4.11), the intensities of α' , α and β subunits were rapidly reduced. After 30 min incubation, these subunits were mostly cross-linked, in agreement with the results reported by Siepaio and Meunier (1995) on cross-linking of β -conglycinin with TGA (7S globulin). The intensity of the corresponding cross-linked proteins was gradually reduced throughout the incubation, suggesting that larger aggregates were formed that were unable to enter the gel.

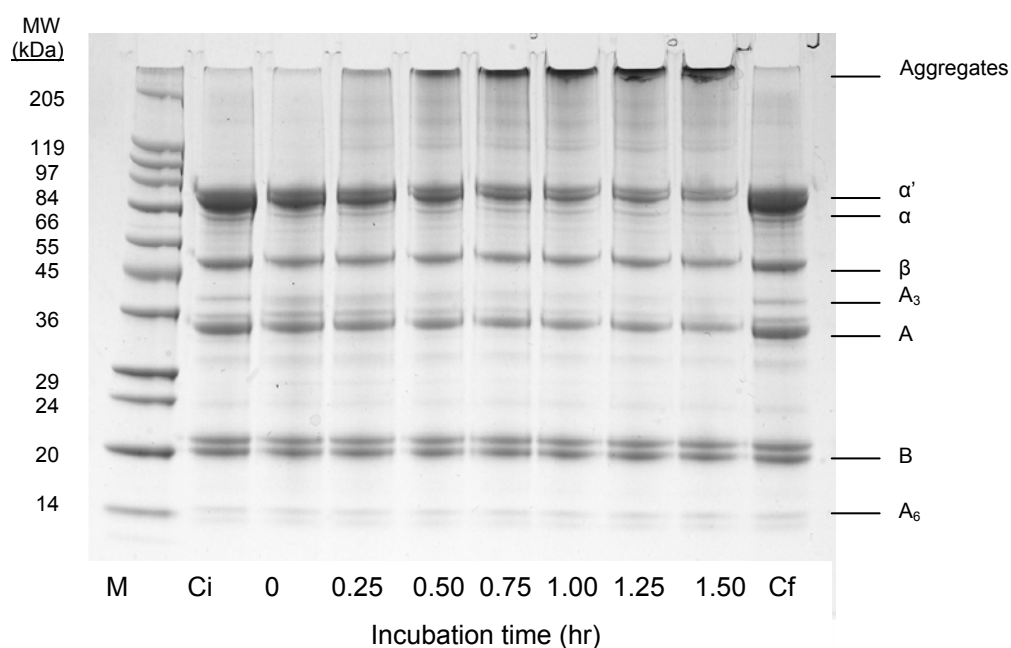


Figure 4.10: A typical SDS-PAGE profile of β -conglycinin incubated with 500 ppm TGA; M = Marker; Ci = Control initial; Cf = Control final; α' , α and β are subunits of β -conglycinin; A and B are the Acidic and Basic subunits of glycinin as a result of cross-contamination. Incubation condition: 2 mg β -conglycinin was dissolved in 1 mL water and incubated with 500 ppm TGA at 20°C. The image is a representative of duplicate gels.

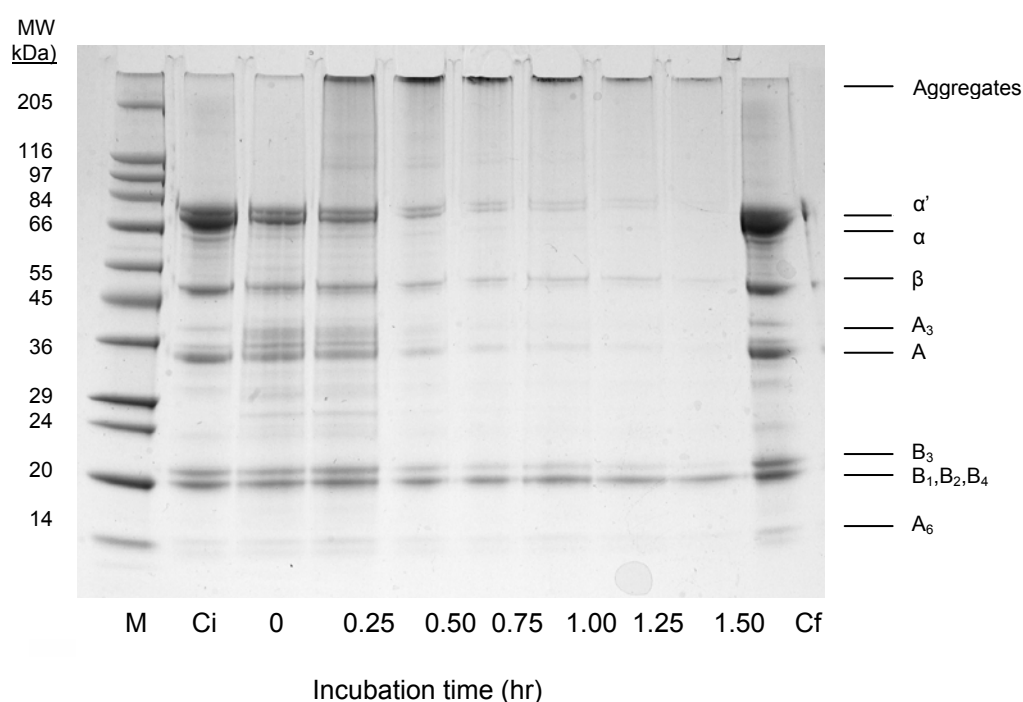


Figure 4.11: A typical SDS-PAGE profiles of β -conglycinin incubated with 1000 ppm TGA at 20 °C; M = Marker; Ci = Control initial; Cf = Control final; α' , α and β are subunits of β -conglycinin. A and B are the cross-contaminated of acidic and basic subunits of glycinin. Incubation condition: 2 mg β -conglycinin was dissolved in 1 mL water and incubated with 500 ppm TGA at 20°C. The image is a representative of duplicate gels.

Since the β -conglycinin fraction was cross-contaminated with A and B subunits of glycinin, the A subunits were also rapidly cross-linked. Within the B subunits, the B₃ polypeptide rapidly cross-linked, but another protein band consisting of B₁, B₂ and B₄ polypeptides, remained unreacted. This profile is similar with the profile observed in defatted soy flour (Figure 4.4), suggesting that the proteins were not fully denatured during extraction conditions used for β -conglycinin.

4.2.3.2 Remaining proteins

The SDS-PAGE profiles in Figures 4.10 and 4.11 were analysed densitometrically. The protein that remained in the incubation solution was quantitated. At $t=0$ min of 500 and 1000 ppm TGA, 9% and 11% proteins were lost due to rapid cross-linking, as presented in Figure 4.12.

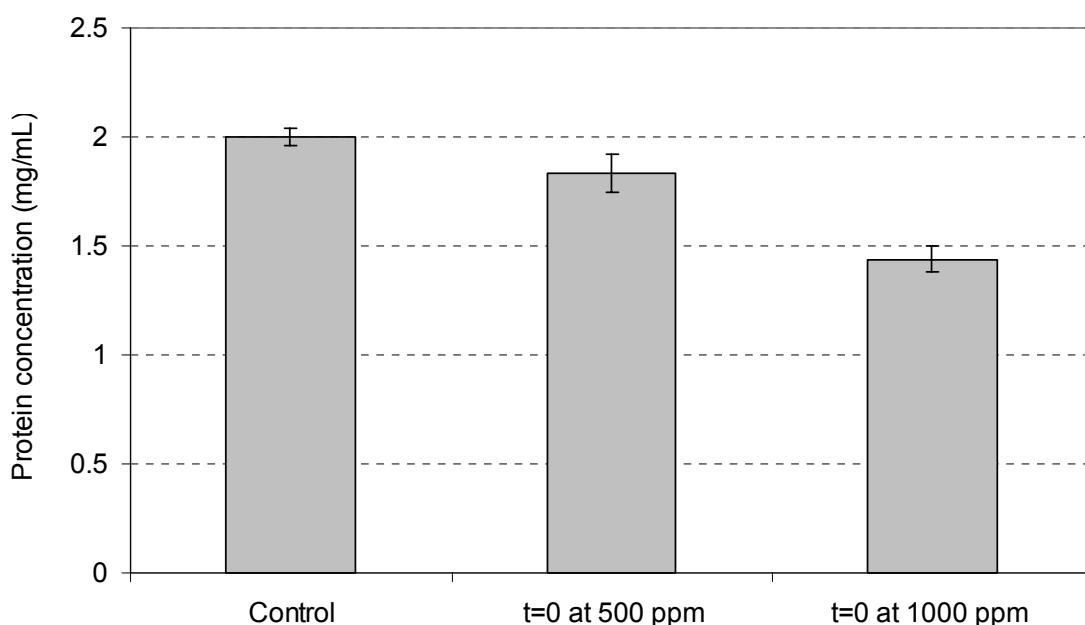


Figure 4.12: Protein concentration in β -conglycinin at $t=0$ of 500 and 1000 ppm TGA. Values are representatives of duplicate measurements. Error bars represent the standard error of the means.

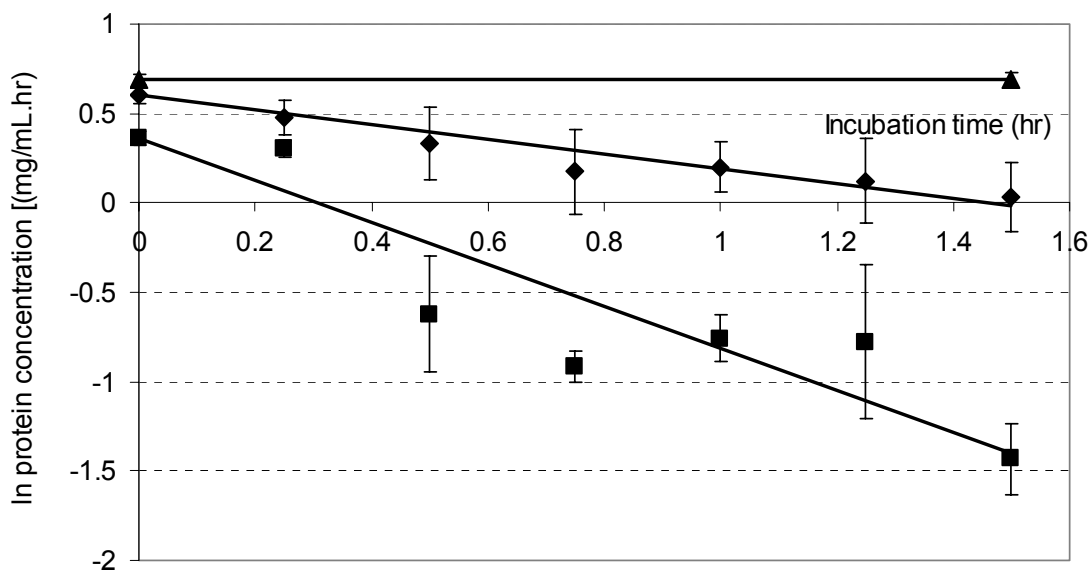


Figure 4.13: Typical linear curves of proteins during incubation of β -conglycinin with TGA at 20°C. Values are representatives of duplicate measurements. Error bars represent the standard error of the means. ◆ = 500 ppm, ■ = 1000 ppm, ▲ = Control.

However, after $t=0$ min, the protein remained in the solution and followed an exponential decrease that fitted to the natural logarithm of the first-order kinetic equation. Typical linear curves are presented in Figure 4.13. As the concentration increased, the slope representing the reaction rate was also increased (Figure 4.14).

4.3 Reaction rates of defatted soy flour and β -conglycinin

The reaction rates of defatted soy flour and β -conglycinin at 500 ppm and 1000 ppm TGA are compared, as presented in Figure 4.14. Since the kinetic analysis of glycinin cross-linking was unable to be determined, the reaction rate of glycinin was quantitated based on the sudden drop of concentration after 1 hr incubation, as presented in Figure 4.15.

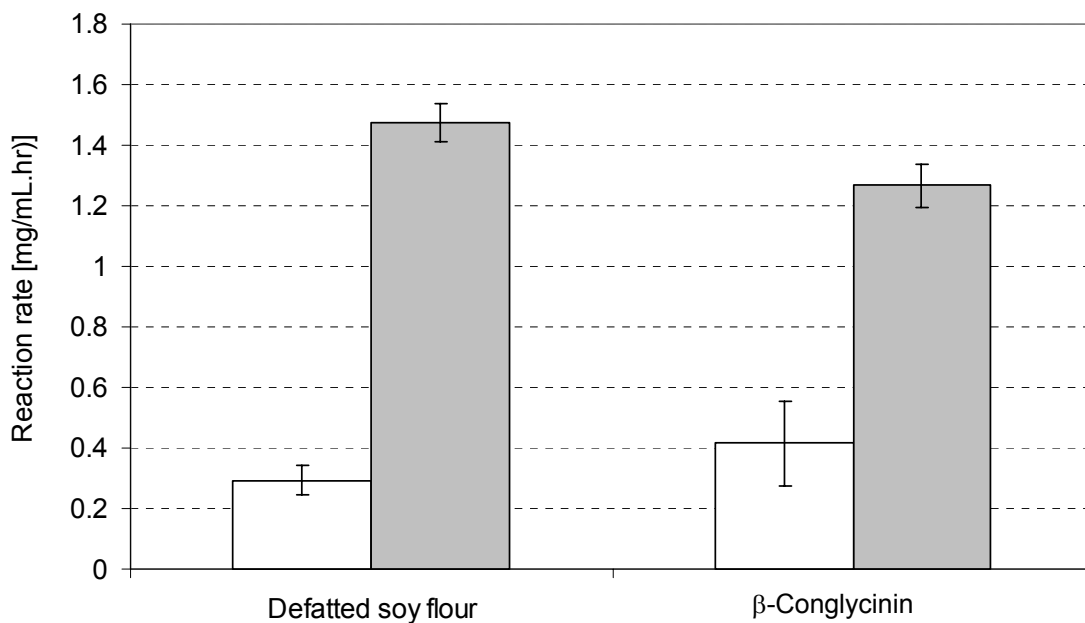


Figure 4.14: The relative rates of defatted soy flour and β -conglycinin incubated with 500 ppm and 1000 ppm TGA respectively. Values are the means of duplicate measurements. The error bars represent the standard error of the means.

Key :- □ = 500 ppm, ■ = 1000 ppm.

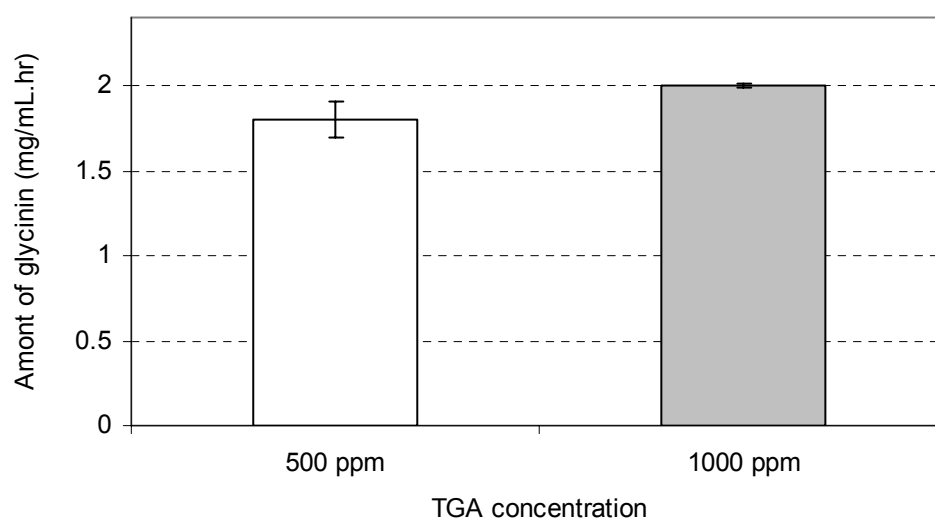


Figure 4.15: The amount of glycinin cross-linked when incubated with 500 ppm and 1000 ppm TGA respectively. Values are the means of duplicate measurements. The error bars represent the standard error of the means.

In all treatments, increasing the TGA concentration from 500 to 1000 ppm resulted in an increase of reaction rate. The results showed that the reactivities of cross-linking in defatted soy flour and β -conglycinin were 5- and 3-fold increased when doubling the TGA concentration. For glycinin, the reactivity of cross-linking was similar for both concentrations.

4.4 Substrate specificities of TGA

4.4.1 Targeted glutamine residues

Soy proteins are known to be good substrates for TGA cross-linking (Ikura et al. 1980, Kang et al. 1994, Tang et al. 2006). This was confirmed in this work. In order to catalyse cross-linking reaction, Coussons et al. (1992a) have proposed two requirements: i) the glutamine must first satisfy an “accessibility” criterion, either by being in a flexible region of polypeptide chain, or by being clearly exposed to solvent in a more structured region of polypeptide chain, ii) in the sequence around the glutamine residue, there must be an absence of features which appear to “discourage” correct interaction with the enzyme.

In the TGA-catalysed cross-linking reaction, the efficiency depends especially on the amino acids that flank the N-terminal side of glutamine (Aeschlimann et al. 1992, Folk and Finlayson 1977, Gorman and Folk 1984, Grootjans et al. 1995, Ohtsuka et al. 2000a, Taguchi et al. 2000, Gorman and Folk 1980, Gorman and Folk 1981). The “bulkiness” of this side chain has been investigated and found to influence the reactivity of proteins at the glutamine residue (Ohtsuka et al. 2000a, Pastor et al. 1999).

For example, by using leucine, a hydrophobic amino acid, in an experiment exploring simple sequences in small peptides, a sequence of leucine-glutamine (-Leu-Gln-) was recognised by TGA rather than glutamine-leucine (-Gln-Leu-) (Ohtsuka et al. 2000a). The glutamine (Q) residue was also active when leucine (L) was present in an extended sequence before Q, for instance LGGQGGG, GLGQGGG or GGLQGGG.

However, when L was in a sequence after glutamine, such as GGGQLGG, GGGQGLG or GGGQQGL, the Q residues in these sequences were not recognised by TGA.

When L in the general series of GLGQGGG and GGLQGGG was replaced by other amino acids, only a few amino acids were recognised by TGA. The hydrophilic amino acids such as arginine (R) and glutamic acid (E), and bulky and large hydrophobic amino acids such as valine (V), phenylalanine (F) and tyrosine (Y) were recognised (Ohtsuka et al. 2000a). Hence, there are a number of possible sequences for TGA cross-linking, as presented in Table 4.2.

Table 4.2: The amino acid sequence around reactive glutamine residue.

GXGQGGG	GGXQGGG
GLGQGGG	GGLQGGG
GRGQGGG	GGRQGGG
GEGQGGG	GGEQGGG
GVGQGGG	GGVQGGG
GFGQGGG	GGFQGGG
GYGQGGG	GGYQGGG
GLGQGGG	GGLQGGG

X = Substituted amino acid in a general sequence.

Adapted from Ohtsuka et al. (2000a).

Sequences such as R-Gln-Phe and Leu-Phe-Gln54-Ile in the primary structure of α -lactalbumin, were well-recognised by TGA (Hayashi et al. 1997, Ohtsuka et al. 2000a). Aeschlimann et al. (1992) reported that when hydrophilic residues such E and R in the vicinity of a reactive glutamine residues, they were cross-linked.

Pastor et al. (1999) reported that when positive charged amino acids were two or four residues away from the glutamine residue towards the N-terminal side, this glutamine residue was reactive and recognised by TGA. However, when the glutamine residue were placed at the N- or C-terminal of the peptide, or placed between two positive charged amino acid residues, or placed between two proline residues, these glutamine residues were not recognised by TGA.

Therefore, the most favourable condition for glutamine residues to react are: i) the position of amino acid residues towards the N-terminal; and ii) types of amino acid residues before glutamine. If V, L, F, E, S or Y appear in the sequence immediately before glutamine then the protein is most reactive (Gorman and Folk 1984, Ohtsuka et al. 2000a). However, the position of F and I at N-terminal position is also important.

As evidenced by SDS-PAGE described earlier, soy proteins were substrates for TGA. In order to examine substrate specificities of soy proteins, amino acid sequences of protein subunits from the National Center for Biotechnology Information (NCBI) data base were obtained. The amino acids of interest around reactive glutamines are K, S, V, L, F, Y, I and E.

4.4.2 Amino acid sequences of protein subunits

β -Conglycinin: The amino acid sequences in the primary structure of α' , α and β subunits of β -conglycinin are presented in Figure 4.13, 4.14 and 4.15. The amino acids of interest are lysine (K), serine (S), valine (V), leucine (L), phenylalanine (F), tyrosine (Y), isoleucine (I) and glutamic acid (E).

In the α' subunit sequence (Figure 4.13), most of the amino acids flanked before the glutamine residues were recognised by TGA. They were positioned at one to four away from the glutamine residue. At least 31 glutamine residues are predicted to be reactive.

In the α subunit (Figure 4.14), the recognised amino acids flanked before glutamine residues were also recognised by TGA. They are within the four position away from glutamine residues. At least 27 glutamine residues were reactive from a total of 49 residues.

In the β subunit (Figure 4.15), the sequence of amino acids before glutamine residues were recognised by TGA. It is predicted at least 20 out of 33 glutamine residues were reactive.

MMRARFPLLLLGVVFLASVSVSFGIAYWE**K**QNPSHN**K**CLRSCNSE**K**DSYRN
 QACHARNLL**K**VEEEEECEEGQIPRPRPQHPERERQQHG**E**KEEDEGEQPRPF
 PFPRPRQPHQ**EEE**HEQ**K**EEHEWHR**K**EE**K**HGG**K**GSEEEQDEREHPRPHQPHQ
KEE**K**HEWQH**K**Q**K**HQG**K**ESEEEEEEDQDEDEEQD**K**ESQSESGSESQREPR
 RH**K**N**K**NPFHFNS**K**RFQTLF**K**NQYGHVR**V**LQRFN**K**RSQQQLQNLRDYRILEFN
SKPNTLLLPHHADADYLIVILNGTAILTLVNDDRDSYN**L**QSGDALRVPAGTT
 YYVVNPDNDENLRMITLAIPVN**K**PGRFESFFLSSTQAQQSYLOGFS**K**NILEAS
 YDT**K**FEEIN**K**VLFGREEGQQQGEER**L**QESVIVEIS**K**KQIRELS**K**HAKSSSR**K**T
 ISS**E**D**K**PFNLRSDPIYSN**K**LG**K**LFEITPE**K**NPQLRDLDVFLSVVDMNEGALF
 LPHFNS**K**AIVVLVINEGEANIELVGI**K**EQQQRQQQEEQPLEVR**K**YRAELSEQ
 DIFVIPAGYPVVVNATSDLNFFAFGINAENNQRNFLAG**S**KNVISQIP**S**QVQ**E**
LAFPGSA**K**DIENLI**K**SQSESYFVDAQ**P**Q**K**EEGN**K**GR**K**GPLSSILRAF**Y**

Figure 4.13: The amino acid sequence of α' subunit of β -conglycinin (NCBI accession no. BAE44299). Letters in bold are the amino acid of interest surrounding the reactive glutamine residues (Q). The **Q** residues are reactive glutamines. The underlined amino acids are the amino acid sequence for TGA recognition. **K** is reactive lysine.

MMRARFPLLLLGLVFLASVSVSFGIAYWE**K**ENP**K**HN**K**CLQSCNSERDSYRNQ
 ACHARCNLL**K**VE**K**EECEEGEIPRPRPRPQHPER**E**PQQPGE**K**EEDEDEEQPRPF
 FPRPQPRQ**EEE**HEQ**K**EEQEWPR**K**EE**K**RGE**K**GSEEEDEDEDEEQDERQFPFPR
 PPHQ**K**EE**K**QEEDEDEEQQQE**S**ESESESELRRH**K**N**K**NPF LFSGSNRFETLF**K**N
QYGRIR**V**LQRFNQRSPQLQNLRDYRILEFN**S**KPNTLLLPNHADADYLIVILNG
 TAILSLVNDDRDSYR**L**QSGDALRVPSGTTYVVVNPDNNENLRLITLAIPVN**K**
 PGRFESFFLSSTEAQQSYLOGFSRNILEASYDT**K**FEEIN**K**VLFSREEGQQQGE
QR**L**QESVIVEIS**K**EQIRALS**K**RA**K**SSSR**K**TISSE**D**KPFNLRSDPIYSN**K**LG**K**FF
 EITPE**K**NPQLRDLDIFLSIVDMNEGALLPHFNS**K**AIIVLVINEGDANIELVGL
KEQQQEQQQEEQPLEVR**K**YRAELSEQDIFVIPAGYPVVVNATSNLNFFAIGI
 NAENNQRN**F**LAG**S**QDNVISQIP**S**QVQ**E**LAFPGSAQAVE**K**LL**K**NQRESYFVDA
QP**K**K**K**EEGN**K**GR**K**GPLSSILRAF**Y**

Figure 4.14: The amino acid sequence of α subunit of β -conglycinin (NCBI accession no. BAE 44298). Letters in bold are the amino acid of interest surrounding the reactive glutamine residues (Q). The **Q** residues are reactive glutamines. The underlined amino acids are the amino acid sequence for TGA recognition. **K** is reactive lysine.

VCVSL**K**VREDENNPFYLRSSN**SFQTLFENQ**NGRIRLL**QRFNKR**SPOLENLRD
 YR**IVQFQSK**PNTILLPHHADADFLLFVLSGRAILTLVNNDDRDSYNLHPGDAQ
 RIPAGTTYLVNPHDHQNL**KIK**LAIPVN**K**PGRYDD**FFLSSTQAQQSYLOGFS**
 HNILETSFHSEFEEINRVLFGE**EEEEQRQQEGVIVELSKEQIRQLS**RR**AKSSSRK**
 TISSEDEPFNLRSRNPIYSNNFG**KFFEITPEK**NPQLRDLDIFLSSVDINEGALLL
 PHFNS**K**AIVILVINEGDANIELVGI**KEQQQKQKQEEEP**LEVQRYRAELSEDD
 VFVIPAAYPFVVNATSNLNFLAFGINAENNQRNFLAGE**K**DN**VVRQIERQVQE**
 LA**FPGSAQDVERLLKKQ**RESYFVDAQP**QQKEEGSKGRK**GPFP SILGALY

Figure 4.15: The amino acid sequence of β subunit of β -conglycinin (NCBI accession no. BAE 02728). Letters in bold are the amino acid of interest surrounding the reactive glutamine residues (Q). The **Q** residues are reactive glutamines. The underlined amino acids are the amino acid sequence for TGA recognition. **K** is reactive lysine.

Glycinin: The glycinin fraction consisted of six A polypeptides and four B polypeptides. The A and B polypeptides are linked by a disulfide bond (Staswick et al. 1984a). Although there were at least six different linkages between A and B polypeptides (Nielson 1985), the amino acid sequence from A₂B_{1a} polypeptides is used as a representative model for analysing the amino acid sequence of glycinin (Staswick et al. 1984b). A₂ representing an A polypeptide, whilst B_{1a} representing a B polypeptide. The amino acid sequences are presented in Figure 4.16 and 4.17.

The A₂ (Figure 4.16) and B_{1a} (Figure 4.17) polypeptides consisted of 37 and 14 glutamine residues. The lysine content in A₂ and B_{1a} polypeptides are 12 and 6 units. B_{1a} polypeptide has the least amount of glutamine and lysine compared to the other subunits. The lower amount of glutamine and lysine residues restricted rapid cross-linking by TGA (Lee et al. 2002).

In the A₂ polypeptide (Figure 4.16), most of the amino acids flanked before glutamine residues were recognised by TGA and they were one to four position away from glutamine residues. At least 18 out of 37 glutamine residues are predicted to be reactive.

REQAQQNECOIQ**K**LNAL**K**PDNRIESEGGFIETWNPNN**K**PFCAGVALSRCTL
 NRNALRRPSYTNGPQ**E**IYIQGNGIFGMIFPGCPSTY**O**EP**O**ESQQRGR**S**QRPQ
 DRHQ**K**VHRFREGDLIAVPTGVAWWMYNNEDTPVVA**V**SIIDTNSLENQ**L**DQM
 PRRFYLAGNQ**E**Q**E**FL**K**YQQQQQGG**S**Q**S****K**G**K**Q**Q**EEENE**G**SNILSGFAPEFL
KEAFGVNMQIVRN**L**QGENEEEDSGAIVTV**K**GGLRVTAPAMR**K**P**Q**Q**E**EDDDD
EE**Q**PQCVETD**K**GCQR**S****K**

Figure 4.16: The amino acid sequence of A₂ (acidic) polypeptide of glycinin (NCBI accession no. P04405). Letters in bold are the amino acid of interest surrounding the reactive glutamine residues (Q). The **Q** residues are reactive glutamines. The underline amino acids are the amino acid sequence for TGA recognition. **K** is reactive lysine.

In the B_{1a} polypeptide (Figure 4.17), the reactive glutamine residues are also flanked by recognise amino acids before N-terminal. At least 11 out of 14 glutamine residues are recognised by TGA. Therefore, judging by the shown sequences, A and B subunits were good substrate for TGA cross-linking. Indeed, when the disulfide bonds were reduced, A and B subunits were more reactive than the other subunits as shown by electrophoretic profile in Figures 4.7 and 4.8.

IDETICTMRL**R**Q**N**IGQ**N**SSPD**I**YN**P**QAGSITTATSLDFPALW**L****K**LSAQY**G**SLR
KNAMFVPHYTLNANS**I**IYALNGRAL**V**Q**V**NCNGERVFDG**E**LQ**E**GGV**L**IV**P**Q
NFA**V**AA**K**SQSDN**F**EY**V**S**F****K**TNDRPSIGNLAGANSLLNAL**P**EE**V**IQHTFN**L****K****S**
QQARQ**V****K**NNNPFSFLVPPQ**E****S****Q**

Figure 4.17: The amino acid sequence of B_{1a} polypeptide of glycinin (NCBI accession no. P04405). Letters in bold are the amino acid of interest surrounding the reactive glutamine residues (Q). The **Q** residues are reactive glutamines. The underline amino acids are the amino acid sequence for TGA recognition. **K** is reactive lysine.

4.4.3 Sequencing around lysine residues

Beside reactive glutamine residues, the sequence around lysine residues also influences the cross-linking reaction by TGA. A number of favourable sequences around the amino donor lysine have been identified as presented in Table 4.3. It was also reported that the presence of N, F, Y and R at N-terminal can restrict the TGA accessibility to the lysine (Ohtsuka et al. 2000b, Taguchi et al. 2000).

Table 4.3: The amino acid sequence around the amine donor lysine.

Amino acid sequence around lysine (K)
<p>-LGKGSOH, -LGKLOH, -LGKROH, -LGKFOH -LGKROH, -ALAKGOH, -ALFKGOH, -ALLKGOH -ALVKGOH, -ALWKGOH, -PSKGOH, -PSKVOH -PLKGOH, -LRKGOH</p>

Adapted from Groenen et al. (1994) and Grootjans et al. (1995).

Based on this table and the amino acid sequences in Figures 4.13-4.17, most of the lysine residues were predicted to be reactive for TGA-catalysed cross-linking.

4.4.4 Reactivities of unfractionated soy proteins

In the unfractionated soy proteins, the B subunits were the least susceptible to cross-linking compared to other subunits, as shown in Figure 4.4. This was in agreement with the results reported by (Tang et al. 2006, Zhang et al. 2003). However, this was not observed in the fractionated glycinin, where the A and B subunits were equally reactive (Figures 4.7 and 4.8). The lack of reactivity was due to the effect of secondary and tertiary structure of the proteins. Glutamine residues in the proteins are cross-linked by TGA if they are located at the end of β -sheet or in unstructured regions (Coussons et al. 1991). Exposing the glutamine residues at the surface of the proteins or in highly flexible region of polypeptide chain enhanced the cross-linking reactivity (Coussons et al. 1992a, Sato et al. 2001, Nieuwenhuizen et al. 2004).

When soy proteins were denatured during extraction, the reactivities of glycinin and β -conglycinin were changed. The glycinin fraction was found to more reactive than β -conglycinin. In the fractionated glycinin, the disulfide bond have been reduced by 2-mercaptoethanol during the extraction (Figure 2.2). By the reduction of disulfide bonds, the protein unfolded making the glutamine residue available to be a substrate for TGA (Coussons et al. 1992b, Nieuwenhuizen et al. 2003). Therefore, the reactivity of B subunits increased.

4.5 Conclusion

The cross-linking reactivities of defatted soy flour, glycinin and β -conglycinin increased with increasing TGA concentration. All subunits were good substrates for TGA cross-linking as shown by SDS-PAGE profiles. As a single fraction, glycinin had a higher reactivity than β -conglycinin. All these findings will be validated in tofu (Chapter 6).

4.6 References

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CHAPTER 5

METHOD DEVELOPMENT: A STANDARD PROCEDURE FOR TOFU MANUFACTURE IN THE LABORATORY

5.1. Background

In Chapters 3 and 4, soy proteins were shown to undergo cross-linking reactions with both glutaraldehyde and transglutaminase (TGA) *in vitro*. Glutaraldehyde changed the degree of aggregation over a range of temperatures and concentrations. Similarly, the two concentrations of TGA investigated resulted in different degrees of reaction. It was thus predicted that introducing these cross-linking agents during tofu manufacture *in situ*, would affect the texture of the final product. Since tofu is prepared by altering soy protein properties at the molecular level, for example by heat denaturation, it was expected that changing the soy protein properties at the molecular level would lead to changes at the macro level, assuming that other components of soy did not interfere with the cross-linking chemistry. In wheat proteins, for example, the changes in wheat proteins at the molecular level following cross-linking resulted in significant changes in the texture of bread and croissants (Gerrard et al. 2003a, Gerrard et al. 2003b, Rasiah 2002). However, before investigating the effect of cross-linking reactions on tofu texture, the development of a standard method of tofu manufacture in the laboratory was a prerequisite in order to minimise tofu variability.

Several studies have investigated the effect of various laboratory scale tofu preparation methods on tofu texture (Byun et al. 1995, Cai et al. 1997, Mujoo et al. 2002). The textural properties of tofu are governed by two main factors: i) the quality of the raw material, such as soybean cultivar or soybean storage period; and ii) how the tofu is manufactured (Cai et al. 2002, Cai and Chang 1998, Poysa and Woodrow 2002). However, the commercial manufacturing process is always subjected to modification to

produce tofu with a desired texture. Some of the modifications that have been applied during tofu manufacturing that affect the textural properties of tofu are summarised in Table 5.1. However, none of these studies investigated changes to the soy proteins at the molecular level.

Table 5.1: Previous studies of modifications in tofu-manufacture and their impact on the texture of tofu.

Investigator	Type of modification	Effect on tofu
Chang et al. (2003)	Compared GDL and calcium sulfate as coagulants. Added chitosan in both GDL and calcium sulfate tofu.	GDL resulted in softer tofu than calcium sulfate. Gel strength increased with higher concentration of chitosan.
Karim et al. (1999)	Added carragenan to calcium sulfate tofu.	Hardness of carragenan-treated tofu decreased compared to non-treated tofu.
Lim et al. (1990)	Compared GDL and calcium sulfate coagulants.	Hardness and firmness of calcium sulfate tofu. lower than GDL tofu.
Shen et al. (1991)	Compared GDL and calcium sulfate coagulants.	Hardness and firmness of GDL tofu higher than calcium sulfate tofu.
Soeda et al. (1995)	Added TGA during tofu manufacture.	TGA increased breaking strength of retorted and non-retorted tofu.

GDL = Glucono- δ -lactone; TGA = Transglutaminase.

These laboratory scale tofu investigations (Table 5.1) utilized a number of mould designs to prepare the tofu. The textural properties, including hardness, were

measured and subsequently correlated with the various factors involved in preparation (Byun et al. 1995, Cai et al. 1997, Min et al. 2005, Mullin et al. 2001). Thus, tofu texture can be altered through modification of the ingredients added during tofu manufacturing. However, none of these investigations looked directly at the cross-linking reaction in soy proteins. It seems likely that the textural property of tofu could be modified by protein cross-linking. Moreover, tofu's acceptability to the consumer depends on its texture, which in turn depends on the soy protein gel (Poysa and Woodrow 2002, Tezuka et al. 2000).

The various laboratory scale tofu moulds have not been tested for their reliability and efficiency. In this study, the methods tofu preparation of Cai et al. (1997), and a mould design of Byun et al. (1995) were adapted. The mould design by Byun et al. (1995) was selected since it produced a uniform tofu geometry. Thus, these two methods are consolidated into a single method to produce tofu tailored to laboratory cross-linking studies. The main objective of this chapter was to establish a standard laboratory tofu manufacturing procedure.

Before a standard procedure of tofu manufacture could be developed, it was necessary to devise a method for measuring textural properties against which the reliability of the tofu manufacturing method could be determined. To achieve this objective, the following experiment was designed with three different steps:

i) Establishment of standard conditions for measuring tofu texture using an Instron Universal Testing Machine (UTM). The physical conditions affecting the measurements were taken into consideration, such as the effect of lubrication or lack of it, the sample shape and height, and compression speed. Any changes in these parameters may affect the measured parameters of tofu, such as fracture force and compression modulus.

ii) Establishment of a standard method for manufacturing tofu from raw soybeans. Numerous factors were involved, including the tofu mould, which could directly affect the texture of the tofu. Soft to firm tofu can be prepared depending on the water content (Liu 1997). The development of the tofu mould design, and the tofu production process in general, was carried out to produce a tofu as consistent as

possible. An important feature of the mould design was to ensure that a standard tofu was produced which possessed a texture similar to firm tofu.

iii) Assessing the physical properties and mass balance of tofu. The parameters include density, volume, weight, moisture content, rheology, and protein content. The rationale behind assessing mass balance was to determine where the soy proteins were partitioning, whether they were going to tofu, whey or both fractions, and whether this partitioning was influenced by cross-linking reagents.

5.2. Establishment of Instron Universal Testing Machine conditions

5.2.1 General background

This was the first step in the development of a standard procedure of tofu preparation. Firm (Chinese-type) tofu was used as a trial sample to establish the most appropriate conditions to analyse the textural properties using an Instron UTM fitted with a General Purpose Interface Bus (GPIB) allowing computer control of the instrument. An in-house program written in LabVIEW version 7.11 (National Instrument, Austin, TX, USA) controlled the probe speed. Data were generated from the Instron UTM and analysed by a Microsoft (MS) Excel Visual Basic Application (Crop & Food Research, Lincoln, New Zealand) for texture profile analysis (TPA).

A large variety of empirical rheological methods are used to assess the texture of a wide range of foods, including apples, cheeses and starch gels (Luyten et al. 1992, Meullenet and Gross 1999). A compression test was used to assess the empirical rheological behaviour of the tofus. In order to obtain an empirical measurement, compressive testing is often used to deform food materials. This compression test involves placing the tofu between two plates and driving the upper plate downwards, at constant speed, to deform the tofu, as illustrated in Figure 5.1. Since the tofu was not bonded with the plates, friction between the plates and the sample can have a significant effect on the measured rheological properties (Canet and Sherman 1988, Chu and Peleg 1985). Thus, the plate surface roughness and the behaviour during compression, such as

the sample releasing its own lubrication, for example water or oil, can influence the measured parameters (Ak and Gunasekaran 1992, Chu and Peleg 1985, Rebouillat and Peleg 1988). A rough surface causes the area of the application of force to vary during the compression test (Chu and Peleg 1985). If there is friction between the plate and the sample, the sample will bulge or “barrel” in the central region during compression, which leads to uneven movement at the compression surface, such as sample sticking or slipping. Thus, a lubricant is normally applied to prevent the sample sticking to the compression surface so that the sample deforms uniformly as it is compressed.

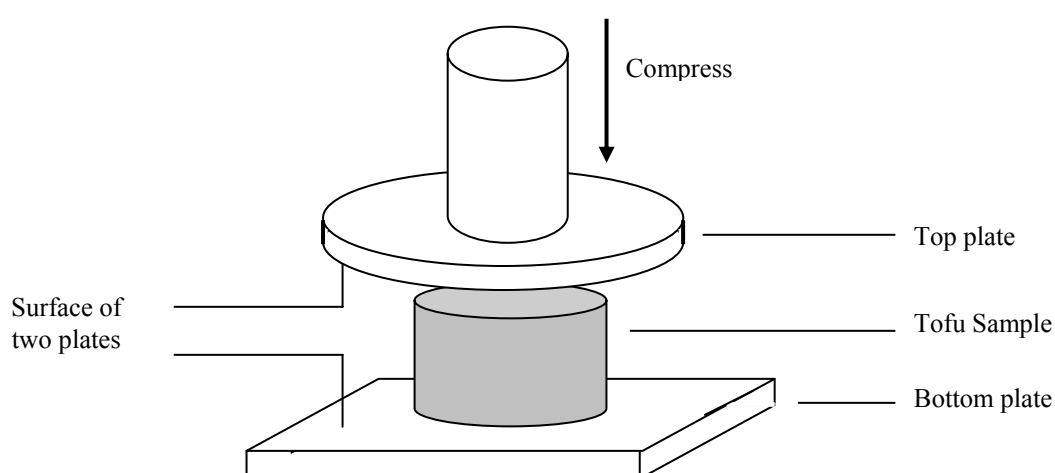


Figure 5.1: Schematic diagram of tofu compression test.

Sample shape and size also affects texture measurement. For example, the measured modulus of deformability of raw potato tissue was found to vary with size and shape (Hicsasmaz and Rizvi 2005). Since the tofu samples are in semi-solid form, a simple geometry such as a cylindrical shape with specific height and diameter is suitable for compression between two parallel plates. Since the tofu gel exhibits some elastic behaviour, the height of the sample must be equal to, or less than, its diameter, otherwise the reliability of compression testing will decrease due to sample buckling (Shaw and Young 1988). Therefore, the height and diameter of the cylindrical shape were fixed at 30 mm and 36 mm respectively.

For uniaxial compression testing, a plunger was attached to the Instron UTM. The plunger was driven downwards at constant speed to compress the sample to 50%

deformation. The texture profile analysis (TPA) generated from the Instron instrument (Bourne 1978) was analysed.

In this test, four compression speeds, 10, 20, 50 and 100 mm/min, were studied. The force of compression against displacement was plotted to give a force-deformation curve as described previously (Bourne 1978, Dobraszczyk and Vincent 1999, Peleg 1987, Sharma et al. 2000). A typical force-deformation curve for tofu is presented in Figure 5.2. An MS Excel Visual Basic Application (Crop & Food Research, Lincoln, New Zealand) was used to determine the compression maximum distance, fracture force and compression modulus (Figure 5.2).

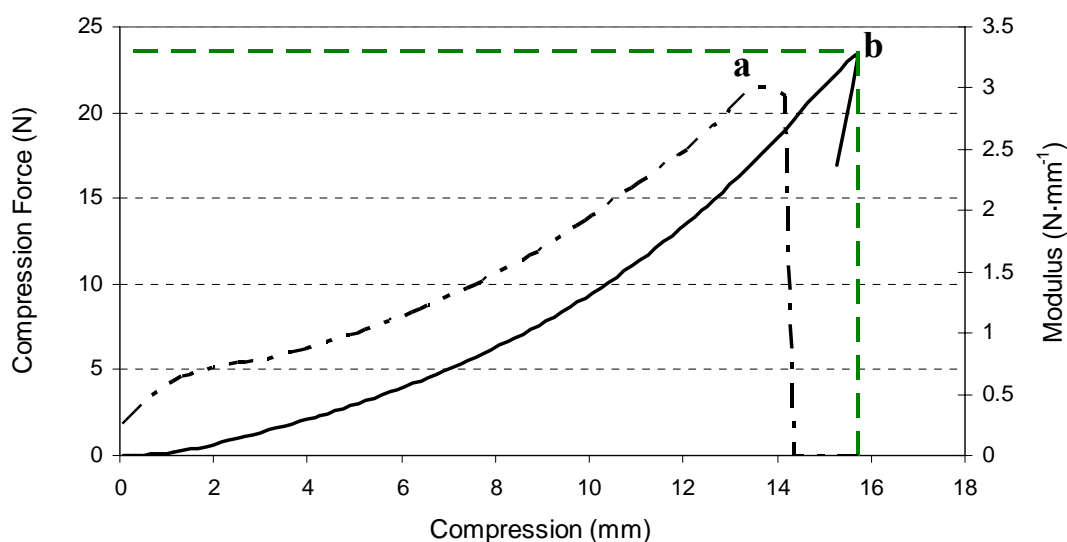


Figure 5.2: A typical tofu force-deformation compression curve. Symbols **a** and **b** represent the maximum compression modulus and fracture point at maximum compression, respectively. — Force; — — Maximum compression; - - - Modulus.

During compression of tofu, the force increased with compression, and, concurrently, the modulus increased to a maximum, before decreasing. The maximum peak of force and modulus are denoted as **a** and **b** (Figure 5.2). The lack of constant initial modulus value indicates that there is either no elastic region or that the region of linear elastic behaviour is very small (Dobraszczyk and Vincent 1999). However, both the fracture force and compression modulus are useful parameters in measuring textural properties (Hicsasmaz and Rizvi 2005, Kang et al. 1991). In the preliminary trial, the fixed parameters and the variables that needed to be fixed are summarized in Table 5.2.

The tofu sample was obtained from commercial (Chinese-type) which had 26 mm height, lower than the height of cylindrical cutter (30 mm). The 26 mm height was considered as full height.

Table 5.2. The fixed parameters and variables that needed to be fixed in the preliminary trial.

Fixed parameters	Value/Measurement
Crosshead size	65 mm diameter
Load cell	50 N
Deformation	50% from the sample height
Cylindrical cutter	36 mm diameter and 30 mm height
Sample shape	Cylindrical with 36 mm diameter and 26 mm height (original height of firm tofu)
Variables that need to be fixed	Value/Measurement
Lubrication with glycerin	With or without glycerine lubrication
Plunger speeds	10, 20, 50 and 100 mm/min
Sample height	26 mm (full height) or 13 mm (half height)

5.2.2 Fixing the variables

Effect of lubrication: Glycerine was used as a lubricant. The effect of glycerine on the fracture force and modulus of a tofu sample (height 26 mm) with various compression speeds are presented in Table 5.3. The means of the fracture force and maximum compression modulus from various compression speeds were compared.

The results in Table 5.3 show that there were no significant differences ($P>0.05$) observed between lubrication and no lubrication on fracture force and compression modulus, suggesting that water released from the tofu acted as a lubricant (Chu and Peleg 1985). However, the standard error of the mean when using glycerine was lower than without glycerine, suggesting less variability in the lubricated sample. Thus, glycerine was applied as a lubricant during compression as an added precaution to ensure that the variability in texture measurements was kept to a minimal level. Furthermore, it was possible that the cross-linked tofu would include samples with

greater roughness than the commercial tofu analysed, or release less water to lubricate during compression.

Effect of sample height: In this study, 26 and 13 mm sample heights were tested to determine the impact of full (26 mm) and half (13 mm) height on the fracture force and compression modulus. The full and half height samples are compared at various compression speeds as presented in Table 5.4.

Table 5.3: The fracture force and compression modulus at various compression speeds with and without glycerine.

Speed (mm/min)	Fracture force		Compression modulus	
	26 – glycerine	26 + glycerine	26 - glycerine	26 + glycerine
10	5.1 ± 0.4	5.2 ± 0.4	0.67 ± 0.06	0.63 ± 0.09
20	4.5 ± 0.4	5.7 ± 0.4	0.66 ± 0.06	0.83 ± 0.03
50	5.9 ± 0.4	5.7 ± 0.4	0.88 ± 0.05	0.80 ± 0.02
100	5.3 ± 0.4	6.1 ± 0.7	0.85 ± 0.03	0.88 ± 0.08
Mean	5.2 ± 0.3 (ns)	5.7 ± 0.2 (ns)	0.76 ± 0.06 (ns)	0.79 ± 0.06 (ns)

Values are the means ± standard error of the mean of four replicate measurements. (ns) = no significant difference ($P > 0.05$) between the mean of glycerine (+) and without glycerine (-) on the fracture force and compression modulus.

Table 5.4: The fracture force and compression modulus at full (26 mm) and half (13 mm) height at various compression speeds lubricated with glycerine.

Speed (mm/min)	Fracture force		Compression modulus	
	26 mm	13 mm	26 mm	13 mm
10	5.8 ± 0.5 (ns)	6.4 ± 0.6	0.96 ± 0.04 (ns)	1.67 ± 0.024
20	5.8 ± 0.6 (ns)	8.1 ± 0.7	1.06 ± 0.11 (ns)	2.21 ± 0.021
50	6.4 ± 0.5 (ns)	8.9 ± 1.1	1.13 ± 0.09 (ns)	2.36 ± 0.027
100	7.1 ± 0.6 (ns)	7.9 ± 0.6	1.16 ± 0.07 (ns)	2.23 ± 0.018
Mean	6.3 ± 0.3(ns)	7.8 ± 0.5(ns)	1.08 ± 0.05 (*)	2.12 ± 0.16 (*)

Values are the means ± standard error of the mean of four replicate measurements. (ns) = no significant difference ($P > 0.05$) on fracture force and compression modulus at compression speeds, and fracture force at 26 and 13 mm height. (*) = The compression modulus in the 26 mm height was significantly different ($P < 0.05$) to the 13 mm height.

On average, the fracture force at 26 mm sample height was not significantly different ($P>0.05$) than the 13 mm sample height. However, the maximum compression modulus at 26 mm sample height was significantly different ($P<0.05$) to the 13 mm sample height. Since the degree of variability of fracture force and compression modulus at 26 mm was less than those at 13 mm, the 26 mm height was chosen as the sample height for all subsequent measurements.

Effect of compression speed: Using 26 mm sample height, the fracture force and compression modulus at various compression speeds in Table 5.4 were analysed to select an appropriate compression speed for subsequent testing. The results showed that there was no significant difference ($P>0.05$) in fracture force as well as compression modulus at various speeds. However, to imitate the “normal” jaw movement as close as possible, the 100 mm/min speed was chosen as the jaw can move up to 4000 mm/min (Tornberg et al. 1985). This speed was also sufficient for the Instron to measure accurately the textural properties of tofu.

Measuring texture: Both fracture force and compression modulus are relevant parameters to measure texture, and provide complementary data. The assessment of texture using TPA imitates jaw movement and provides data related to the fracture behaviour of the tofu sample. This analysis was used to provide a direct measurement of the impact of cross-linking on the tofu texture.

5.3 Establishment of a standard method of tofu manufacture

In order to optimize the laboratory preparation of tofu, two objectives were set:

- i) To determine the optimal compression load and period.
- ii) To improve the tofu mould design.

5.3.1 Optimal compression

The purpose of determining the optimal compression load was to produce tofu with a density as similar to firm (Chinese-type) as possible. The optimal loading period

was determined by compressing the tofu until no further displacement was observed, thus ensuring that the tofu produced would have a stable and consistent density (Cai et al. 1997).

In preliminary experiments, a series of compression loads consisting of 600, 700, 800 and 900 g weights was tested. The 600 and 900 g compression loads were found to have a significant difference in term of surface displacement and were further assessed. The displacement of the top of the tofu surface manufacture using these loads was measured at 5 min intervals over an hour period. The displacement profiles are presented in Figure 5.3.

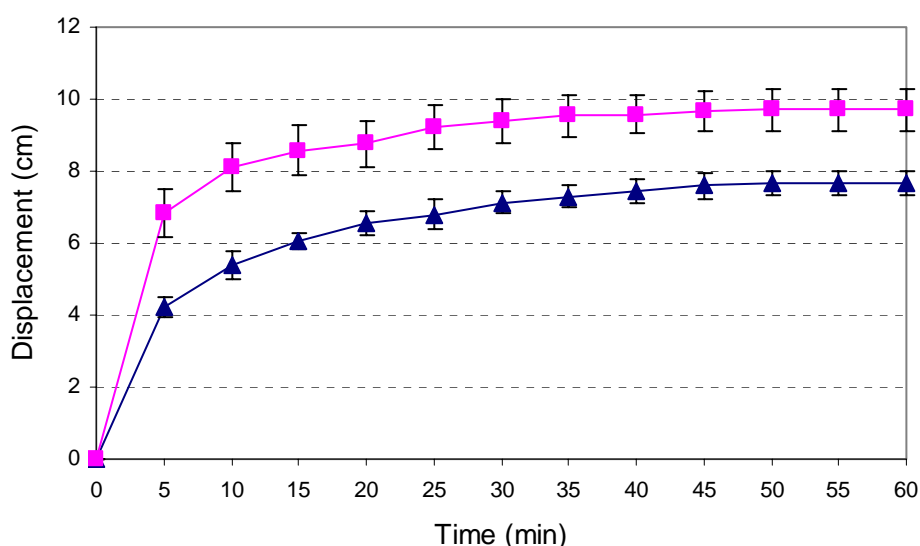


Figure 5.3: Displacement of the top tofu surface using 600 (▲) and 900 (■) g loads. The values are the means of triplicate measurements. Error bars represent the standard error of the means of triplicate measurements. The tofu was prepared according to the method in section 9.4.2 (Chapter 9) and the tofu mould used was adapted from Byun et al. (1995) (Figure 5.4).

As the compression load increased, the final displacement of the top tofu surface increased. Upon increasing the compression load from 600 to 900 g, the time at which the tofu compaction ceased occurred after a shorter period of time, between 5-10 min. After that, the displacement started to reach a plateau at 60 min. Since the 900 g load produced a relatively dense of tofu, in which the strengthening action by the cross-linking reagents may not have been readily detected, the 600 g load was selected. The

600 g load produced tofu more similar to the Chinese type tofu, which had sufficient softness and was likely to be a more sensitive sample for monitoring any change in properties due to cross-linking.

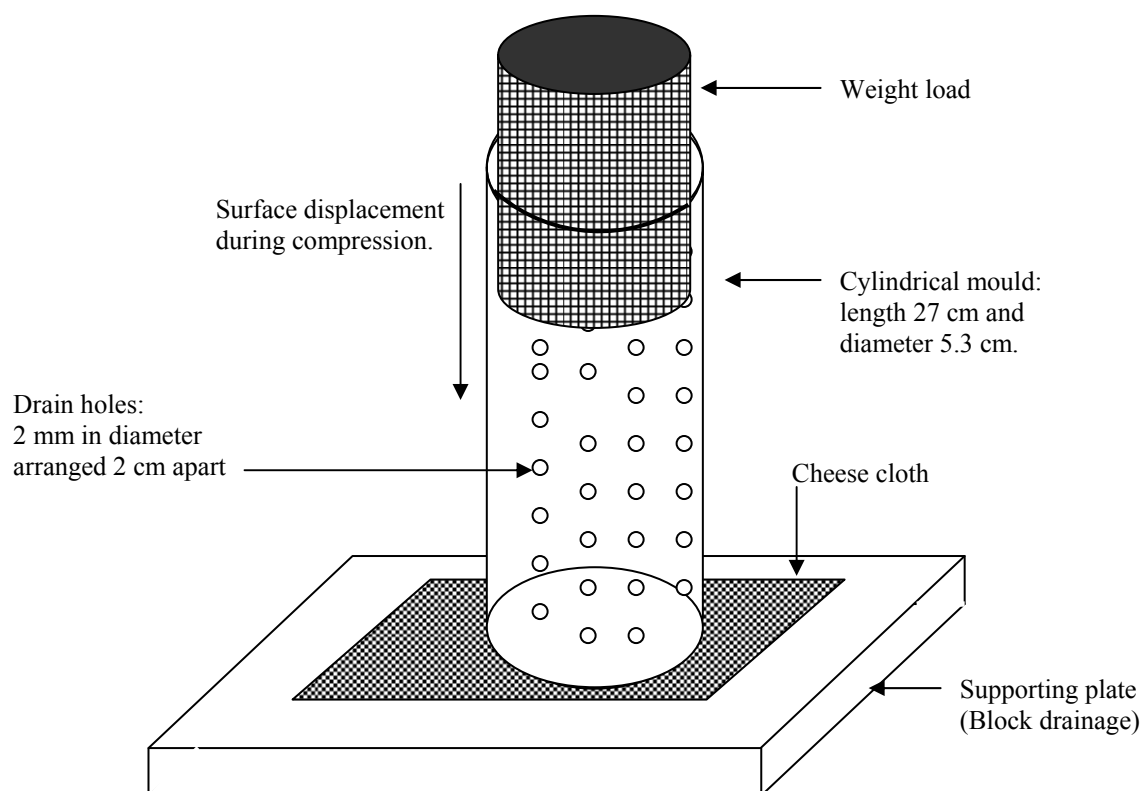


Figure 5.4: Prototype tofu mould adapted from Byun et al. (1995) (First design).

5.3.2 Mould design

The original tofu mould design was based on that of Byun et al. (1995) as shown in Figure 5.4 (first design). The cylindrical mould was made of polyacrylic plastic which had a glossy surface. This prototype design was then modified. A problem with the original design was that the supporting plate has no lower drain hole to squeeze the water, which led to ‘soggy’ tofu at the base of the mould. This resulted in non-uniformity in hardness of the tofu across the sample. Tofu prepared from this mould was not used for Instron testing. Thus, this design was improved as schematically presented in Figure 5.5 (second design).

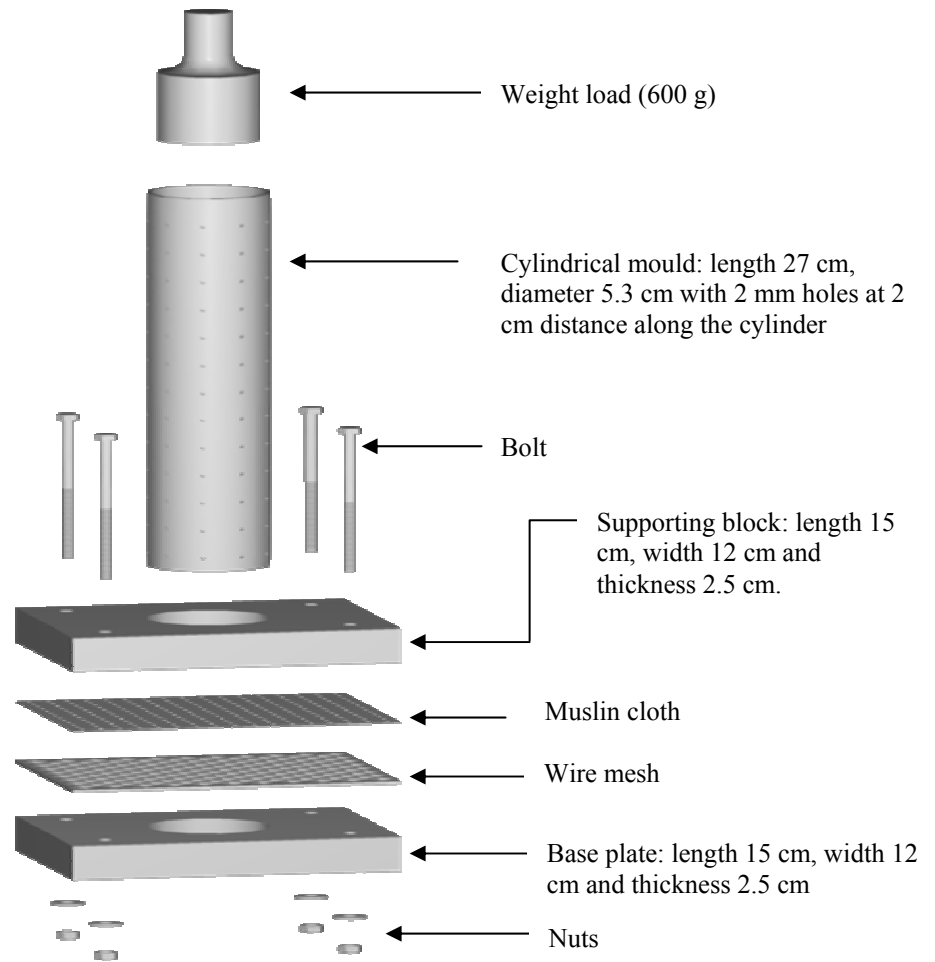


Figure 5.5: The second tofu mould design.

To ensure better uniformity of the fracture force throughout the tofu column from the second design (Figure 5.5), the tofu column was cut equally into three portions namely top, middle and bottom as presented in Figure 5.6. The centre of each cylindrical portion was then cut using a cylindrical cutter to obtain a cylindrically shaped sample of 36 diameter and 30 mm height. The fracture force of each portion was measured as presented in Figure 5.7.

It was found that the fracture force at the top of the tofu mould was not significantly different ($P > 0.05$) from the middle portion. However, the fracture force at the bottom was significantly lower ($P < 0.05$) than both the top and middle portions. These results suggested that the tofu had a non-uniformity fracture force gradient

resulting from non-uniformity in the tofu mould. At the bottom position, the tofu was softened as a result of the blockage of the water drainage holes caused by the thickness of the supporting block. Therefore, to overcome this drawback, the supporting block was replaced with a thin supporting sheet as shown in Figure 5.8 (third design).

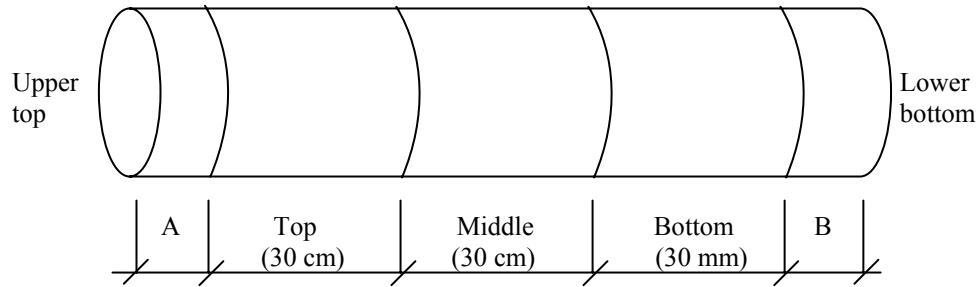


Figure 5.6: The three portions of tofu prior to Instron testing. Portions A and B were cut off.

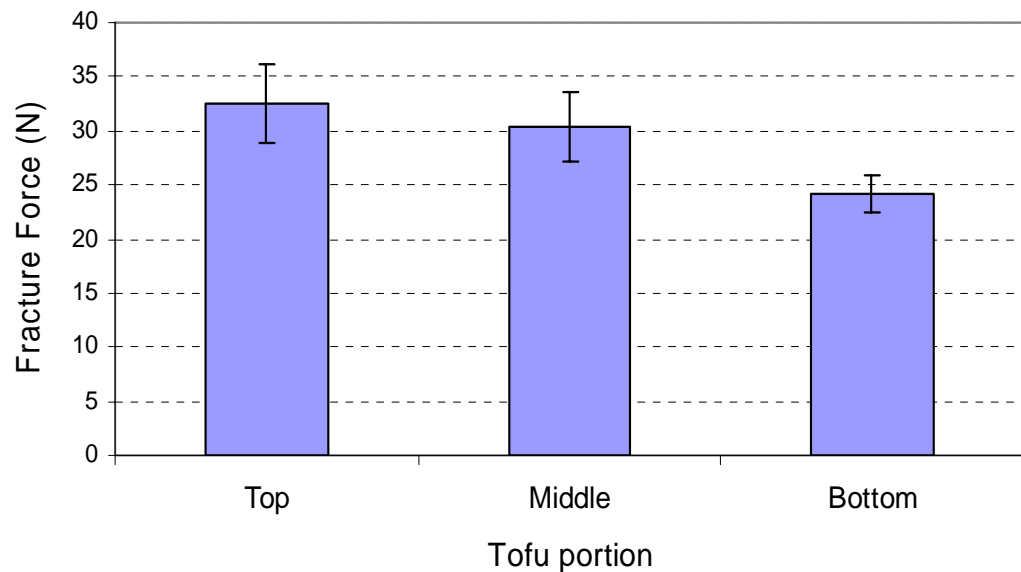


Figure 5.7: The fracture force of the top, middle and bottom portions of the tofu column from the second tofu mould design. The values are the means of three replicate measurements. Error bars represent the standard error of the mean of triplicate measurements.

The fracture forces of the top, middle and bottom portions of the tofu column were measured as presented in Figure 5.9. The results show that there was uniformity in the fracture force gradient from the top to the bottom of the tofu column. The fracture

force at the top was significantly different ($P<0.05$) than the middle and bottom position. Similarly, the fracture force at the middle was significantly different ($P<0.05$) than the bottom portion. Due to the consistency in fracture force gradient along the tofu column, this design was subsequently used as the standard mould in this study.

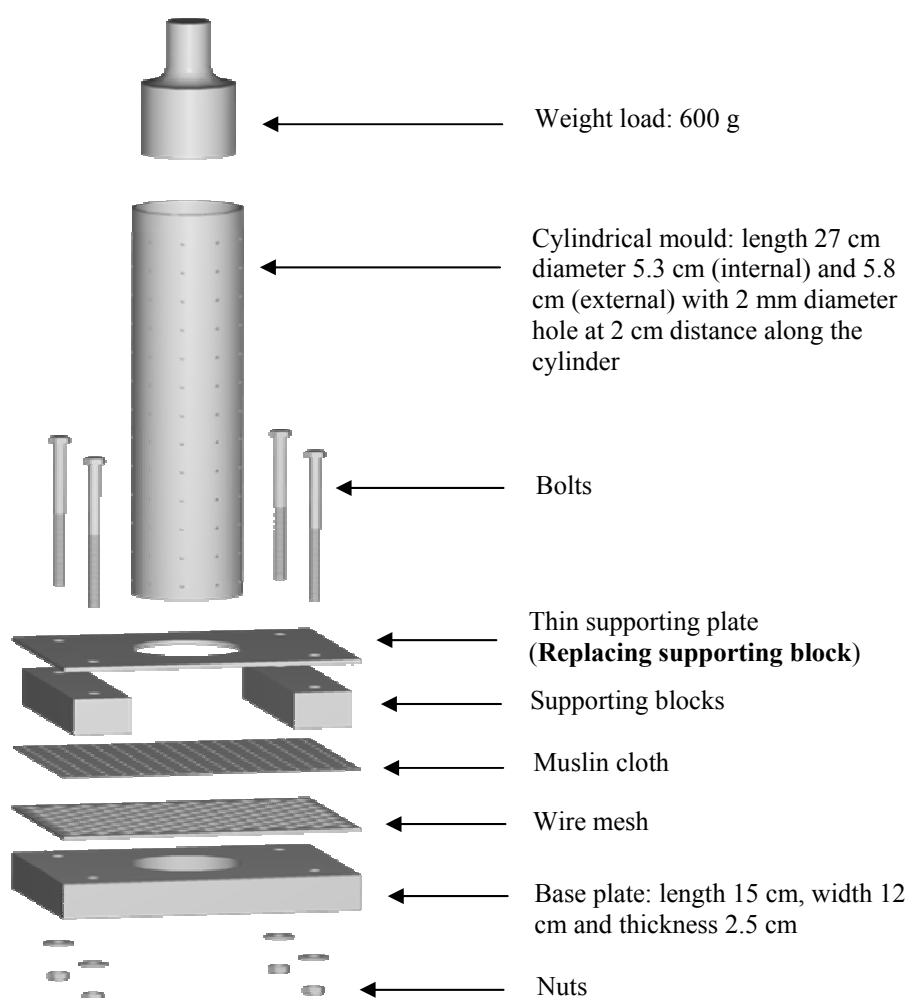


Figure 5.8: The third design of the tofu mould. The supporting block in Figure 5.6 was replaced with a thin supporting plate.

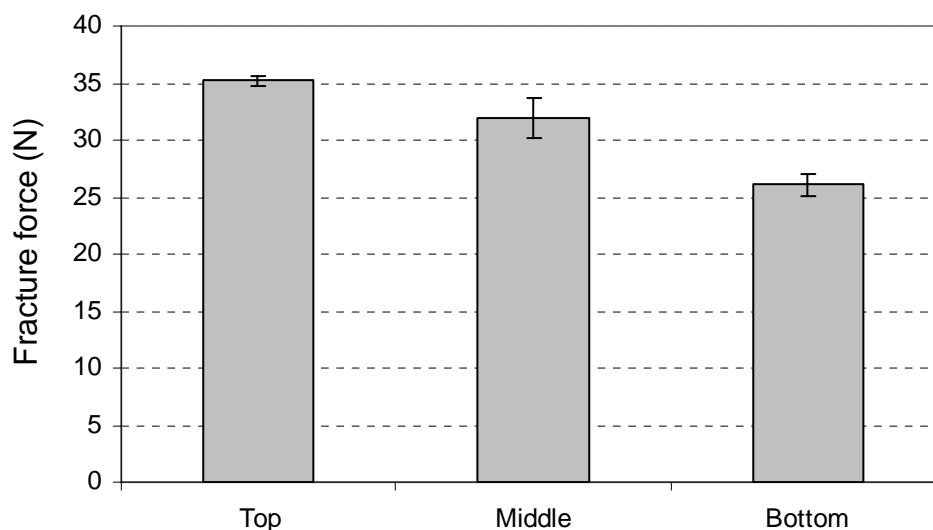


Figure 5.9: The fracture forces of the top, middle and bottom portions of the third tofu mould design. The values are the means of three replicate measurements. Error bars represent the standard error of the mean of triplicate measurements.

5.4 Assessing the mass balance in tofu manufacture

Figure 5.10 illustrates tofu processing, in which the soymilk is converted into tofu and whey. In assessing the mass balance, the key parameter was the protein content, since the protein is partitioned between the tofu and whey. Mass balances also determine the way in which the various cross-linking reactions investigated in this research affected the tofu manufacture process. A standard tofu was prepared as described in section 9.4.2 (Chapter 9), using the third tofu mould design (Figure 5.8) and the mass balance parameters were assessed.

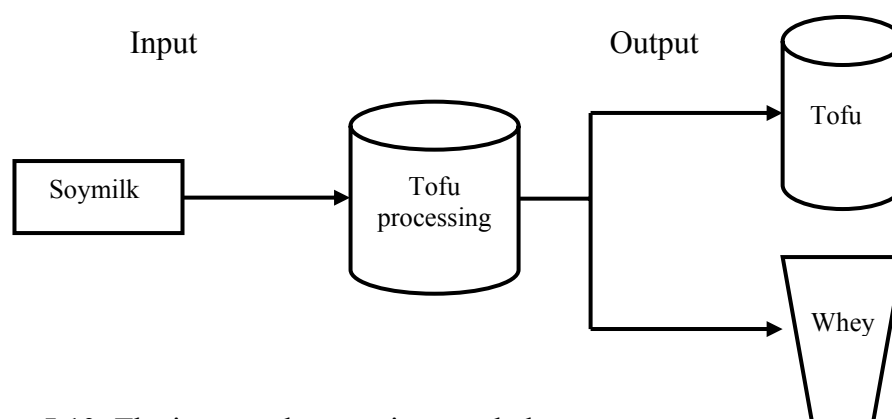


Figure 5.10: The input and output in mass balance assessment.

The volume, weight, water and protein contents were evaluated as presented in Table 5.5. The results in this table show three main points.

- i) The significant reduction ($P < 0.05$) of weight, volume and water content.
- ii) The partitioning of soy proteins from soymilk to tofu and whey.
- iii) The preservation of total protein content.

Table 5.5: The assessment of parameters of the input and output in mass balance.

Parameter	Input	Output		Total output	Reduction (%)
	Soymilk	Tofu	Whey		
Volume (mL)	800 ± 0	245 ± 2	485 ± 2	730 ± 3	9 (*)
Weight (g)	799 ± 1	227 ± 1	493 ± 2	720 ± 1	10 (*)
Moisture content (%)	91 ± 1	78 ± 1	97 (ns)	-	-
Water content (mL)	727 ± 1	178 ± 2	479 ± 2	657 ± 4	10 (*)
Protein content (g)	31.2 ± 0.1	29.4 ± 0.6	1.9 ± 0.2	31.3 ± 0.8	Nil
Protein partitioning (%)	(100%)	(94%)	(6%)	(100%)	Nil
Density (g/mL)	-	1.04 ± 0.02	-	-	-

Values are the means \pm standard error of the means of triplicate measurements. (*) = significant different at $P < 0.05$. ns = no significant different among three replicates.

The 10% reduction in volume and weight of the total output was expected, as this is due to the evaporation of water during soymilk boiling. Meanwhile, the soy proteins in soymilk were partitioned, without loss, between the tofu and whey. About 94% of soy proteins partitioned into the tofu with the remaining 6% in the whey. This result concurs with Kao et al. (2003) observation that most of the denatured soy proteins coagulate to form tofu, while the residue whey is almost completely free from proteins.

In the preliminary investigation of density measurement, the tofu samples were soaked in the water at 20°C for five days in order to determine whether the tofu absorbed water during soaking. Results showed that the tofu did not absorb water during soaking. By measuring a water displacement in the measuring cylinder at 20°C , the density of tofu was 1.04 g/mL , which was greater than water density at 20°C (0.998 g/mL). This density represents the compactness of tofu. Based on these results, the third

tofu mould design produced reliable data and maintained protein content during tofu manufacturing.

5.5 Conclusion

With the establishment of a standard tofu manufacture process, the numbers of variables that influence the texture of tofu has been reduced. The resulting tofu had reproducible properties in which any changes due to the cross-linking reaction could be detected in subsequent experiments. A mass balance analysis demonstrated that the reduction in volume and weight was correlated with the loss of water, without altering the protein content. About 94% of soy proteins were preserved in the tofu. The effect of cross-linking reagents on the mass balance and textural properties of tofu is investigated in Chapter 6.

5.6 References

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CHAPTER 6

THE EFFECT OF GLUTARALDEHYDE AND TRANSGLUTAMINASE ON THE TEXTURAL PROPERTIES OF TOFU

6.1 Background

In the *in vitro* experiments (Chapters 3 and 4), glutaraldehyde and TGA cross-linked soy proteins via the Maillard reaction and enzymatic catalysis. In this chapter, the cross-linking reactions that occurred *in vitro* were validated *in situ*, by adding glutaraldehyde and TGA to tofu and assessing how they changed the texture. An experimental design was set up in an analogous fashion to those in Chapters 3 and 4. In this study, the addition of cross-linking reagent was made at two stages: i) before boiling the soymilk, and ii) after boiling the soymilk. The purpose of adding cross-linking reagents at two different stages was to establish whether addition of cross-links at different stages of food processing led to differences in properties. Before boiling, the soy proteins are in their native state, whilst after boiling, they are denatured (Kohyama et al. 1995). In the native state, tofu does not form, even after adding coagulant (Liu 1997). On the other hand, denatured soy proteins do form tofu, after the addition of coagulant. The addition of a cross-linking reagent before and after boiling was therefore of interest.

The experimental design is presented in Table 6.1. Glutaraldehyde was chosen as the cross-linking reagent because it was found to be more reactive than formaldehyde and glyceraldehyde. Four final concentrations were employed with the purpose of probing whether the degree of change in textural properties correlated with different concentrations used. 1 and 2 mM glutaraldehyde were used based on the results in Chapter 3, whilst 15 and 30 mM glutaraldehyde were used to investigate the effect of higher concentrations on texture. In TGA treatment, concentrations of 1000 and 5000 ppm were employed to determine the effect of enzymatic cross-linking of soy proteins on tofu texture.

Table 6.1: A treatment design for glutaraldehyde- and transglutaminase-treated tofu.

Cross-linking reagent	Treatment	
	Added before boiling	Added after boiling
Glutaraldehyde	1 mM	1 mM
	2 mM	2 mM
	15 mM	15 mM
	30 mM	30 mM
Transglutaminase	1000 ppm	1000 ppm
	5000 ppm	5000 ppm

Each treatment was prepared in five replications.

The effect of the cross-linking reaction on tofu texture was investigated using the following steps. Tofu was manufactured using the method described in section 9.5.1 (Chapter 9) and Figure 5.7 (Chapter 5). A mass balance analysis was carried out to ensure all material was accounted for. The parameters measured were volume, weight, water content and protein content. SDS-PAGE and densitometric analysis were carried out in an analogous manner to Chapters 3 and 4 to obtain information about the degree of cross-linking. The fracture force was measured and compared between the treatments.

6.2 Glutaraldehyde treatment

6.2.1 Mass balance analysis

The moisture content and density: The moisture content of soymilk, tofu and whey, and the density of tofu are summarised in Appendix 1. The moisture content in soymilk and whey were constant at 90% at 1 and 2 mM, and 97% at 15 and 30 mM.

Meanwhile, in tofu, the moisture content ranged from 70% to 80%, and the density ranged from 1.01 to 1.09 g/mL.

The volume, weight and total water content: The total volume, weight and water content of the input and output from all treatments are summarised in Appendices 2, 3 and 4. The volume, weight and total water content of the output were reduced significantly ($P < 0.05$) in the range of 6-11%, 10-13% and 11-13% for all treatments, confirming that these reductions were due to the loss of water.

The protein content: The most important parameter indicating the movement of mass is the protein content. Tables 6.2 and 6.3 summarise the protein content of glutaraldehyde-treated samples. As expected, the results showed that the protein content in the output was not significantly different ($P > 0.05$) to that in the input, although the total water content was significantly reduced. There was no significant difference ($P > 0.05$) in the amount of proteins that partitioned into tofu and whey in the different treatments.

6.2.2 SDS-PAGE profiles

Defatted soybean, soymilk, tofu and whey were analysed by SDS-PAGE. To assess the extent of disulfide bonding, the extraction of proteins and SDS-PAGE was performed under two conditions: i) reduced conditions, using dithiothreitol (DTT) and 2-mercaptoethanol, and ii) non-reduced conditions, omitting dithiothreitol (DTT) and 2-mercaptoethanol. Subsequently, the SDS-PAGE profile was analysed densitometrically to assess the changes.

6.2.2.1 Reducing and non-reducing SDS-PAGE

In the preliminary investigation, protein from defatted soybeans, protein from defatted soymilk and tofu, and protein from whey of control samples were extracted and analysed by reduced and non-reduced SDS-PAGE. The SDS-PAGE profiles are presented in Figure 6.1.

Table 6.2: The protein content of the input and output after treatment with different concentrations of glutaraldehyde.

Treatment	Total input	Output		Total output	Total input versus Total output
	Soymilk (g)	Tofu (g)	Whey (g)	Tofu + Whey (g)	
1. Control	34.46 ± 0.39	32.31 ± 0.47	2.04 ± 0.39	34.35 ± 0.29	ns
2. 1 mM Glut B	35.73 ± 1.47	33.00 ± 1.18	1.74 ± 0.26	34.74 ± 1.41	ns
3. 2 mM Glut B	35.76 ± 2.97	33.37 ± 1.18	1.79 ± 0.02	35.17 ± 1.20	ns
4. 1 mM Glut A	35.18 ± 0.10	32.46 ± 0.03	2.63 ± 0.05	35.09 ± 0.05	ns
5. 2 mM Glut A	35.24 ± 0.95	34.19 ± 0.49	1.61 ± 0.51	35.80 ± 1.00	ns
1. Control	34.46 ± 0.39	32.31 ± 0.47 (a,b)	2.04 ± 0.39	34.35 ± 0.29	ns
2. 15 mM Glut B	34.51 ± 1.16	33.95 ± 0.83 (a)	1.20 ± 0.03 (a,c)	35.15 ± 0.82	ns
3. 30 mM Glut B	34.38 ± 0.99	32.73 ± 1.02 (c)	1.06 ± 0.03 (a)	33.79 ± 1.04	ns
4. 15 mM Glut A	35.21 ± 1.15	33.53 ± 0.84	1.71 ± 0.03 (b,c)	35.24 ± 0.83	ns
5. 30 mM Glut A	35.60 ± 1.06	34.68 ± 0.82 (b,c)	1.17 ± 0.06 (b)	35.85 ± 0.83	ns

Values are the means ± standard error of the means of five replicate measurements. Means in the same column followed by the same letter are significantly different ($P < 0.05$). ns = not significant different ($P > 0.05$). Glut B = Added glutaraldehyde before boiling; Glut A = Added glutaraldehyde after boiling.

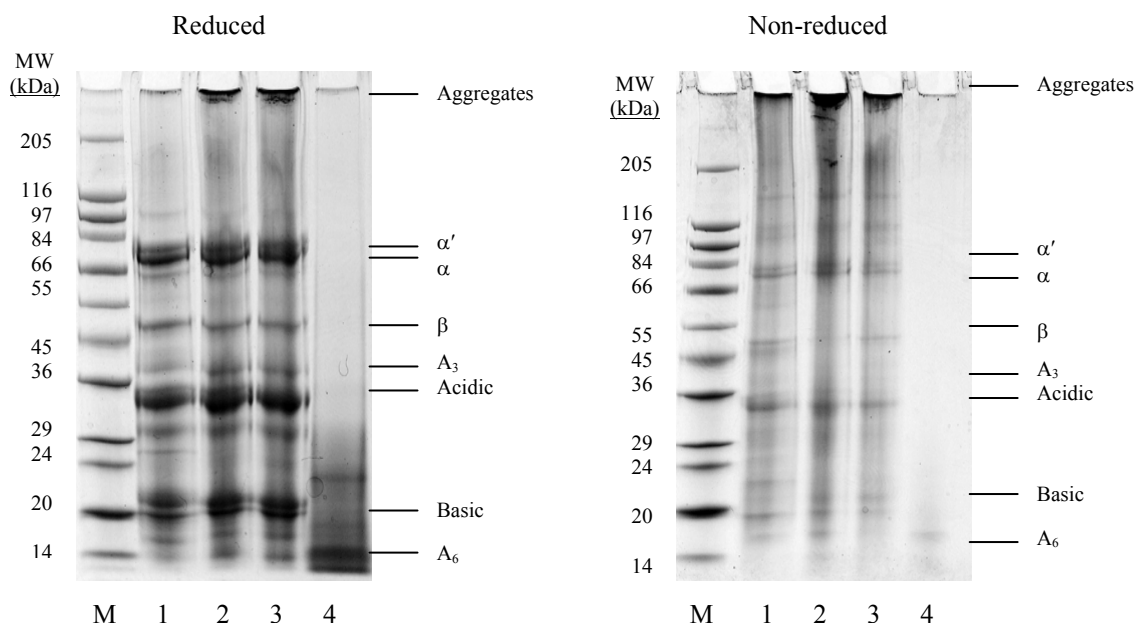


Figure 6.1: SDS-PAGE profile of reduced and non-reduced samples. M = marker; 1 = defatted soybeans; 2 = control soymilk; 3 = control tofu; 4 = control whey; CL = cross-linked proteins. The electrophoretic profiles are representative of duplicate gels.

Under reducing conditions, the electrophoretic profile of defatted soybean shows a good separation of all major subunits of soy proteins (Lane 1), in agreement with those shown in Chapter 2. In the control soymilk and tofu (Lane 2 and 3), despite showing all major subunits, multimeric and aggregated proteins at the top of the gel were also observed. The control whey (Lane 4) shows lower molecular weight protein (<20 kDa) and the most intense bands were present at about 14 kDa, presumed to be the A₆ subunit. This was in agreement the results obtained by Kao et al. (2003).

In contrast, under non-reduced conditions, there is a poor separation of protein subunits of all samples. The electrophoretic profiles also show multimeric and aggregated proteins at the top of the gel. The poor separation in the non-reducing conditions indicates the presence of disulfide bonds in all samples.

The presence of disulfide bonds was expected as the A and B subunits of soy proteins are linked by disulfide bonds (Kitamura et al. 1976, Renkema et al. 2001). The α', α and β subunits of β-conglycinin are also partly associated with disulfide bonds (Utsumi and Kinsella 1985). Moreover, during boiling, proteins are denatured and more

disulfide bonds are formed as a result of self cross-linking by cysteine residues during unfolding (Huang et al. 2003, Kao et al. 2003, Kohyama et al. 1995).

Compared to reducing SDS-PAGE, the non-reducing condition did not facilitate quantification of proteins by densitometry. Therefore, in the following SDS-PAGE analysis, only reducing conditions were employed, allowing specific quantitation of the cross-links introduced by glutaraldehyde and TGA without considering any disulfide bonds.

6.2.2.2 Treatment before boiling

Figures 6.2 and 6.3 represent the electrophoretic profiles of soymilk, tofu and whey from the treatments with different concentrations of glutaraldehyde added before soymilk boiling. The relative concentration of aggregated proteins and protein subunits in soymilk and tofu were quantified densitometrically as presented in Table 6.3.

Soymilk and tofu treated at lower concentrations of glutaraldehyde: The SDS-PAGE (Figure 6.2) of soymilk showed that the intensity of aggregated proteins at the top of the gel increased from 0 to 1 to 2 mM glutaraldehyde. However, the intensity of aggregated proteins in tofu remained unchanged. For the multimeric proteins, represented by region a, the intensity also increased with increasing concentration, but in tofu, the intensity of region a remained unchanged. These results suggest that the increasing intensity of aggregated and multimeric proteins in soymilk was due to the protein cross-linking. The unchanged intensity of aggregated and multimeric proteins observed on tofu was due to the protein-protein aggregation during coagulation that renders cross-linked proteins hard to extract.

In Table 6.3, the relative concentration of aggregated proteins in soymilk at 1 mM glutaraldehyde increased to 180%. In the corresponding subunits, the relative concentration showed a slight reduction. When the concentration of glutaraldehyde was increased to 2 mM, the relative concentration of aggregated proteins was further increased to 251%, and the relative concentration of corresponding subunits was further decreased to 36-76%.

In the corresponding tofus, 1 mM glutaraldehyde resulted in unaltered aggregated proteins and also caused a slight reduction in protein subunits, with the exception of the A_6 subunit. When the concentration increased to 2 mM, the relative concentration of aggregated proteins was increased to 134% at which point the relative concentrations of the corresponding β and A_6 subunits were reduced to 74% and 54%, respectively. The relative concentrations of other subunits remained unchanged.

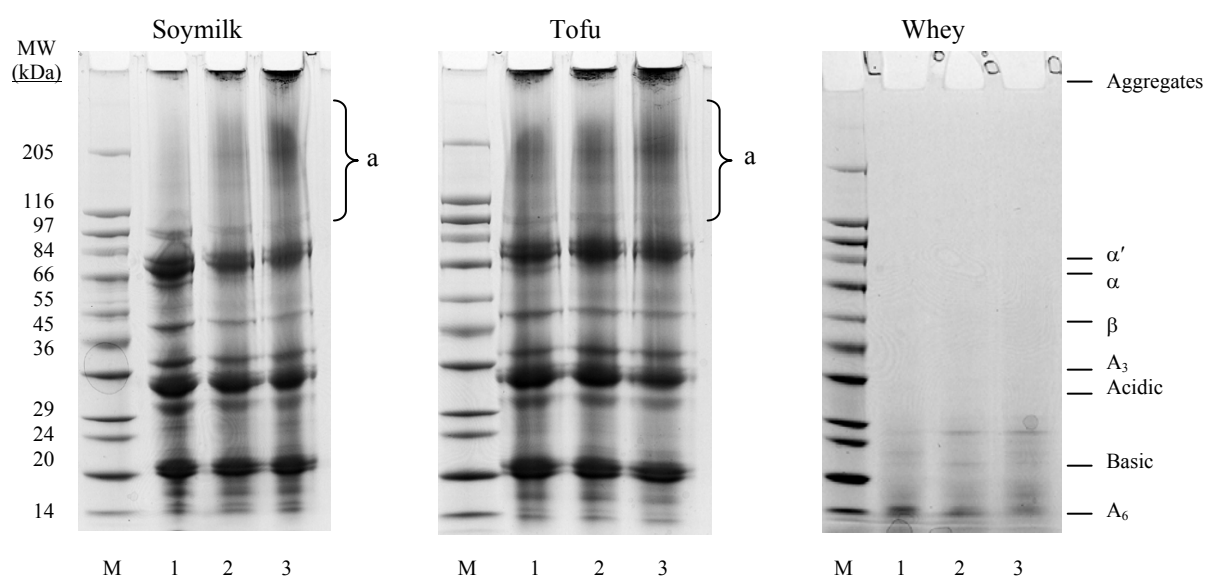


Figure 6.2: SDS-PAGE profile of proteins in soymilk, tofu and whey under reducing condition. 1 and 2 mM glutaraldehyde were added before soymilk boiling. M = marker; 1 = control; 2 and 3 are 1 and 2 mM glutaraldehyde; The electrophoretic profiles are representative of duplicate gels.

Table 6.3: The changes of protein composition in aggregates and subunits following treatment with glutaraldehyde added before boiling the soy milk.

Aggregates and subunits	Relative concentration(% of control)							
	Soymilk		Tofu		Soymilk		Tofu	
	1mMglutB	2mMglutB	1mMglutB	2mMglutB	15mMglutB	30mMglutB	15mMglutB	30mMglutB
Aggregates	180 ± 11	251 ± 22	106 ± 18	134 ± 14	89 ± 21	nd	nd	nd
$\alpha' + \alpha$	94 ± 2	64 ± 1	115 ± 3	96 ± 1	nd	nd	nd	nd
β	94 ± 1	36 ± 2	101 ± 6	74 ± 3	nd	nd	nd	nd
A ₃	106 ± 2	50 ± 3	116 ± 4	97 ± 2	nd	nd	nd	nd
A	89 ± 1	61 ± 2	111 ± 11	96 ± 7	14 ± 1	nd	17 ± 4	nd
B	97 ± 1	76 ± 1	122 ± 10	105 ± 5	45 ± 7	nd	56 ± 5	nd
A ₆	98 ± 16	71 ± 4	96 ± 2	54 ± 4	59 ± 20	nd	45 ± 1	nd

Values are the means ± standard errors of the mean of duplicate measurements. 1mMglutB and 2mMglutB are 1 and 2 mM glutaraldehyde added before soymilk boiling. 15mMglutB and 30mMglutB are 15 and 30 mM glutaraldehyde added before soymilk boiling. nd = non-detectable. The percentage was based on the control soymilk and tofu respectively. Acidic comprised of A₁, A₂, A₄ and A₅ subunits. Basic comprised of B₁₋₄ subunits.

Soymilk and tofu treated at higher concentrations of glutaraldehyde: The SDS-PAGE profile of 15 mM glutaraldehyde treated soymilk (Figure 6.3) showed that the α' , α and β subunits were completely cross-linked. The B, A and A_6 subunits were not fully cross-linked and remained as bands on the gel, although much reduced in intensity compared to the control.

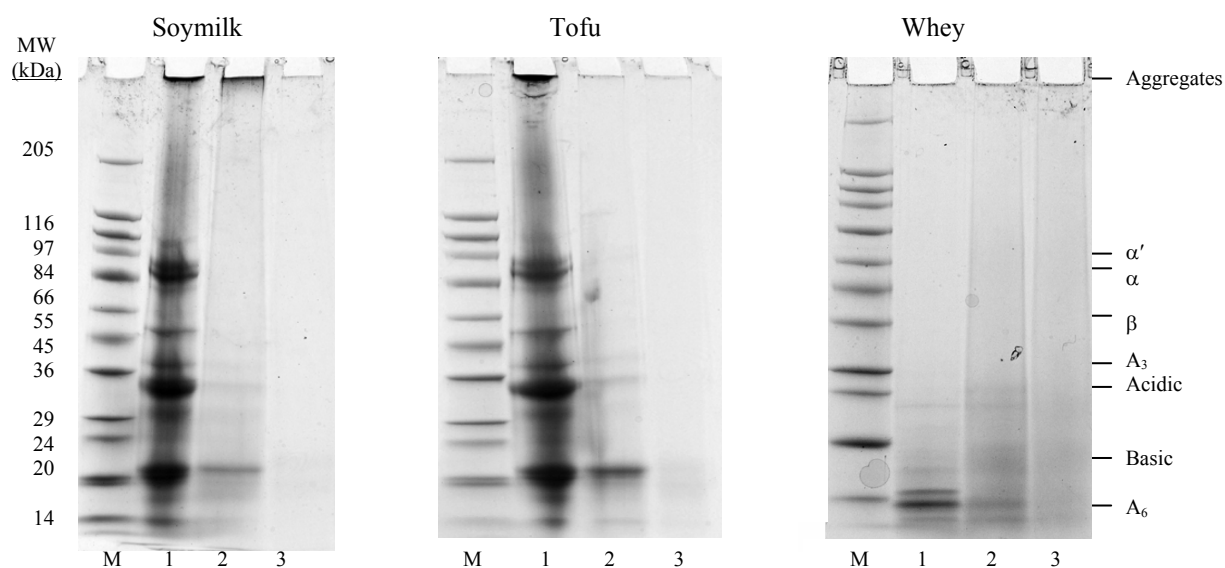


Figure 6.3: SDS-PAGE profile of proteins in soymilk, tofu and whey run under reduced condition. 15 and 30 mM glutaraldehyde were added before soymilk boiling. M = marker; 1 = control; 2 and 3 are 15 and 30 mM glutaraldehyde; The electrophoretic profiles are representative of duplicate gels.

In the corresponding tofu, the SDS-PAGE profile did not show any aggregated proteins, and maintained only the A, B and A_6 subunits. This result confirms that the β -conglycinin is more susceptible to cross-linking than glycinin, in agreement with the *in vitro* results discussed in Chapter 3. In this case, cross-linked proteins were observed in the soymilk but not in tofu, suggesting that the cross-linked proteins in tofu became too big to enter the gel as a result of highly aggregated cross-linked proteins. When the concentration of glutaraldehyde was increased to 30 mM, no protein bands were detected in either soymilk or tofu.

In Table 6.3, when the soymilk was treated with 15 mM glutaraldehyde, the relative concentration of the aggregated proteins and the corresponding subunits of A, B

and A₆ subunits were reduced to 89%, 14%, 45% and 59%, respectively. In the corresponding tofu, the aggregated proteins were not observed. However, the intensity of A, B and A₆ subunits were further reduced to 17%, 56% and 45%. These results confirmed that these subunits were less reactive than those of α' , α and β subunits of β -conglycinin, which was consistent with *in vitro* incubation (Chapter 3).

Whey: The whey of the control tofu showed a group of lower molecular weight proteins present around 14 kDa, which is consistent with the profiles reported by Kao et al. (2003). When 1 and 2 mM glutaraldehyde were employed, this group of proteins remained detected. However, when the concentration increased to 15 and 30 mM, this group of proteins was not observed, suggesting that the smaller proteins were cross-linked, in agreement with the lower protein content measured in Tables 6.2 and 6.3.

6.2.2.3 Treatment after boiling

The effect of 1, 2, 15 and 30 mM glutaraldehyde added after soymilk boiling on the SDS-PAGE profiles of soymilk, tofu and whey are presented in Figures 6.4 and 6.5. Since glutaraldehyde was not added to the soymilk during boiling, the densitometric analysis was only carried out on the tofu. The relative concentration of aggregated proteins and corresponding subunits are presented in Table 6.4.

Tofu treated at lower concentrations: The electrophoretic pattern in tofu treated with 1 mM glutaraldehyde (Figure 6.4) shows that the relative concentration of cross-linked protein was increased to 130%, whilst the relative concentration of subunits decreased to 80-90%. Increasing the concentration to 2 mM resulted in a further increase in aggregated protein concentration to 178% with a concurrent decrease in subunit concentration to 50-80%. Among the subunits, α' , α and β subunits were the most reduced, indicating that these subunits were more highly susceptible than the other subunits, consistent with earlier results from *in vitro* incubation (Chapter 3). Compared with glutaraldehyde treatment before boiling (Figure 6.2), α' , α and β subunits were more reactive when treated with glutaraldehyde after boiling, suggesting that in the denatured state, the lysine and arginine residues were more available than in the native state for Maillard-type cross-linking.

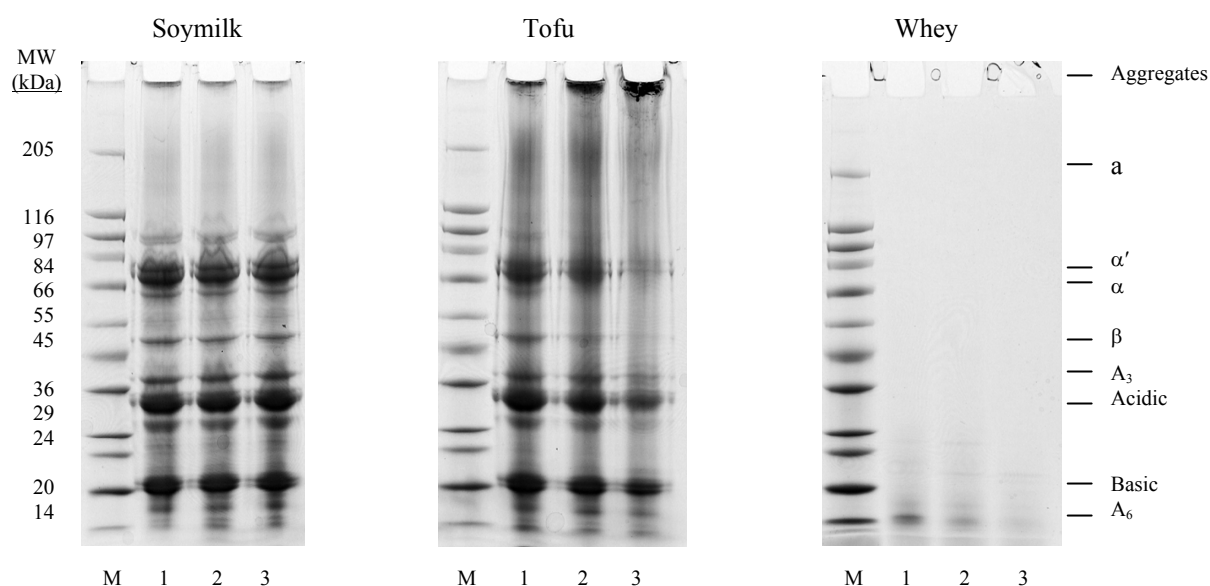


Figure 6.4: SDS-PAGE profile of proteins in soymilk, tofu and whey under reducing condition. 1 and 2 mM glutaraldehyde were added after soymilk boiling. M = marker; 1 = control; 2 and 3 are 1 and 2 mM glutaraldehyde; The electrophoretic profiles are representative of duplicate gels.

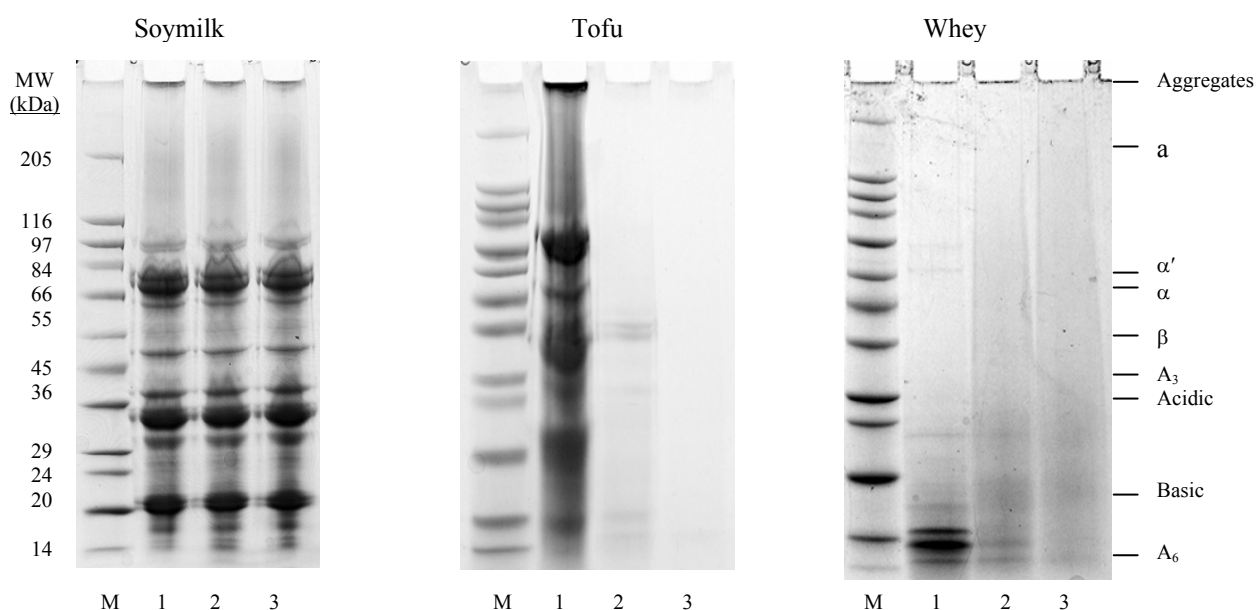


Figure 6.5: SDS-PAGE profile of proteins in soymilk, tofu and whey under reducing condition. 15 and 30 mM glutaraldehyde were added after soymilk boiling. M = marker; 1 = control; 2 and 3 are 15 and 30 mM glutaraldehyde; The electrophoretic profiles are representative of duplicate gels.

Table 6.4: Relative concentration of aggregated proteins and protein subunits following treatment with glutaraldehyde after boiling.

Aggregates and subunits	Relative concentration (% of control)			
	Tofu			
	1 mM	2 mM	15 mM	30 mM
Aggregates	130 ± 30	173 ± 28	23 ± 1	17 ± 3
$\alpha' + \alpha$	88 ± 13	51 ± 3	nd	nd
β	78 ± 7	49 ± 3	nd	nd
A ₃	84 ± 4	64 ± 4	nd	nd
A	83 ± 3	70 ± 1	18 ± 1	nd
B	85 ± 1	79 ± 3	nd	nd
A ₆	81 ± 1	70 ± 13	nd	nd

Values are the means ± standard error of the means of duplicate measurements. 1 mM and 2 mM are 1 and 2 mM glutaraldehyde added after soymilk boiling. 15 mM and 30 mM are 15 and 30 mM glutaraldehyde added after soymilk boiling. nd = non-detectable. The percentage was based on the control soymilk and tofu respectively. Acidic comprised of A₁, A₂, A₄ and A₅ subunits. Basic comprised of B₁₋₄ subunits.

Multimeric proteins were also observed on the gel, represented by region a. However, their intensities, which were more intense in control and 1 mM treatment, became less intense in the 2 mM treatment, indicating that they were cross-linked to form larger aggregates that were unable to enter the gel.

Tofu treated at higher concentrations: Using higher concentrations of glutaraldehyde, faint A and A₆ subunits were detected on the gel. The other subunits were absent. The absence of subunits and aggregated proteins in both 15 and 30 mM treatments suggests that these subunits were highly aggregated to form very large cross-linked aggregates that were insoluble in the extracting buffer.

Whey: Electrophoretic profiles from whey treated at all concentrations (Figure 6.4 and 6.5) showed that a group of lower molecular weight proteins was detected around 14 kDa in various intensities. Using 1 and 2 mM glutaraldehyde, these bands started to disappear. When 15 and 30 mM glutaraldehyde were employed, these bands

were not detected. These results suggest that smaller proteins were cross-linked forming larger aggregates. Reduced concentrations of lower molecular proteins observed at higher concentrations were consistent with the low protein content in Table 6.3.

6.3 The textural properties of glutaraldehyde-treated tofu

The textural properties of glutaraldehyde-treated tofu were measured as previously described (Chapter 5). The fracture force is the key parameter to describe texture, as it is closely related to fracturability during chewing of food materials (Tornberg et al. 1985). The harder the tofu, the greater the work required to break down the internal bonding (Huang et al. 2003). The compression modulus is related to the stiffness of visco-elastic materials such as tofu (Sharma et al. 2000, Visser 1991). Earlier studies reported that soy proteins that had different compositions of glycinin and β -conglycinin, or different subunits in the glycinin fraction, resulted in tofu of different texture (Cai et al. 2002, Kohyama et al. 1995, Schaefer and Love 1992, Tezuka et al. 2000).

Fracture distance: Fracture distance is the distance at which fracture of tofu occurs. The fracture distance of all treatments is presented in Table 6.6. Results showed that the fracture distance for tofu treated with the low concentrations of glutaraldehyde was in a range of 15.55-15.67 mm. For the higher concentrations, the fracture distance was in the range of 12.38-15.65 mm, indicating that the deformation of some of the tofu samples required a lower fracture force.

Table 6.6: The fracture distance of glutaraldehyde-treated tofu samples.

Glutaraldehyde (mM)	Added before boiling	Added after boiling
0	15.27 ± 0.36	15.27 ± 0.36
1	15.55 ± 0.04	15.67 ± 0.06
2	15.64 ± 0.04	15.61 ± 0.06
15	13.82 ± 0.60	15.64 ± 0.03
30	12.38 ± 0.28	15.65 ± 0.02

Values are the means ± standard error of the means of five replicate measurements.

Fracture force at different concentrations: The fracture force of tofu samples treated with concentrations of glutaraldehyde, and added at before and after soymilk boiling is presented in Figures 6.6. In the treatment before boiling, the addition of 1 mM glutaraldehyde resulted in no significant difference of fracture force ($P>0.05$) to the control. However, when the concentration was increased to 2 mM, the fracture force increased significantly ($P<0.05$) from 33 to 39 N. The increased fracture force was attributed to the formation of covalent bonds that strengthened the protein network (Olde Damink et al. 1995). Comparison of fracture force profiles and SDS-PAGE profiles reveal that the fracture force increased when a small number of proteins were cross-linked.

In contrast, by using higher concentrations of glutaraldehyde, the fracture force at 15 mM was significantly reduced ($P<0.05$) to 20 N. Increasing concentration to 30 mM, the fracture force was further reduced significantly ($P<0.05$) to 15 N. In general, a high degree of cross-linking observed on the gel (Figure 6.3) resulted in more “brittle” tofu.

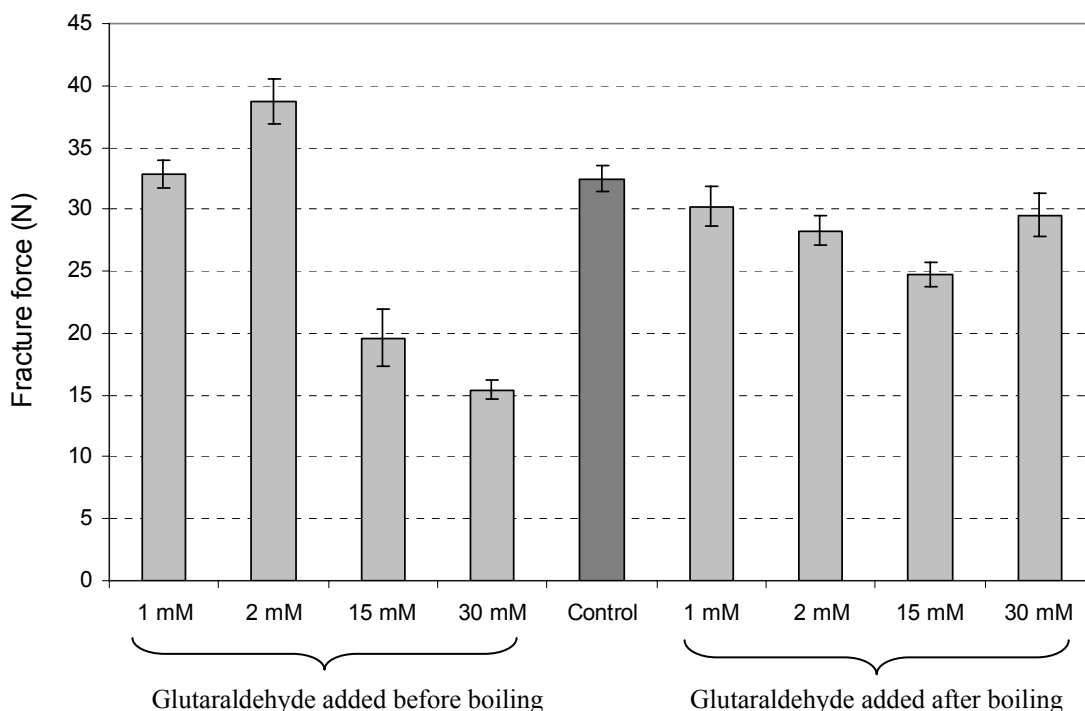


Figure 6.6: The fracture force of different concentrations of glutaraldehyde-treated tofu. Values are the means five replicate measurements. Error bars represent the standard error of the means.

In the treatment after boiling, the fracture force started to decrease at 1 mM glutaraldehyde. Increasing the concentration to 2 mM, the fracture force was further reduced significantly ($P < 0.05$) to the control. In the SDS-PAGE profile (Figure 6.4), the reduction in fracture force corresponded to an increase of cross-linked subunits.

At higher concentrations, the fracture force at 15 mM glutaraldehyde was further reduced ($P < 0.05$). However, when the concentration was increased to 30 mM, the fracture force increased to a similar value to the 1 mM treated sample, in contrast to the before boiling treatment.

Compression modulus: The compression modulus of samples was assessed in order to corroborate the measurements of fracture force and these data are presented in Figure 6.7. In the before boiling treatment, the compression modulus profile follows the fracture force profile. The compression modulus of 1 mM glutaraldehyde-treated tofu was not significantly different ($P > 0.05$) to the control. However, the compression modulus was significantly increased ($P < 0.05$) when the concentration was increased

from 1 to 2 mM. The compression modulus is related to the stiffness of the sample (Visser 1991). With a higher compression modulus indicating higher stiffness and firmness of tofu (Huang et al. 2003). Meanwhile, by using higher concentrations, the compression moduli were clearly decreased significantly ($P < 0.05$) to the control, indicating that higher concentrations produced tofu that were less firm and stiff, particularly when added before soymilk boiling.

In the after boiling treatment, the addition of lower concentrations resulted in lowering compression modulus significantly ($P < 0.05$) compared to the control. With increased concentrations, 15 mM further reduced the compression modulus significantly ($P < 0.05$), but with 30 mM, the compression modulus had no significant difference ($P > 0.05$) to the control. All treatments of glutaraldehyde showed a compression modulus in a range of 3.0–3.3 N/mm. Therefore, regardless of the concentration employed, the compression modulus did not vary to a large degree, in contrast to the before boiling treatment.

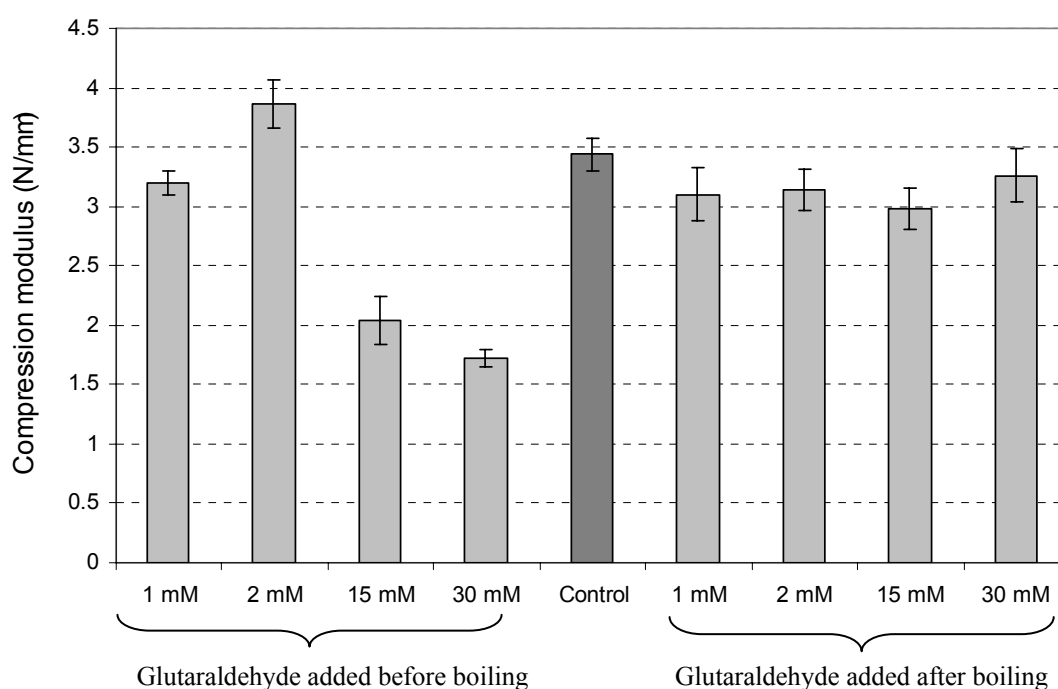


Figure 6.7: The compression modulus at different concentrations of glutaraldehyde. Values are the means five replicate measurements. Error bars represent the standard error of the means.

Relationship between fracture force and moisture content: Changes in fracture force are thought to have a strong relationship with moisture content. Kehagias et al. (1995) reported that higher moisture content contributed to a lower fracturability. To examine this relationship, the fracture force against the moisture content was plotted for all concentrations of glutaraldehyde, added before and after soymilk boiling. They are presented in Figure 6.8 and 6.9.

There was not a strong correlation between the fracture force and the moisture content with treatment either before or after boiling soymilk. However, the general trend indicated that as the moisture content increased, the fracture force decreased. The drop in fracture force with treatment before boiling was faster than that for treatment after boiling.

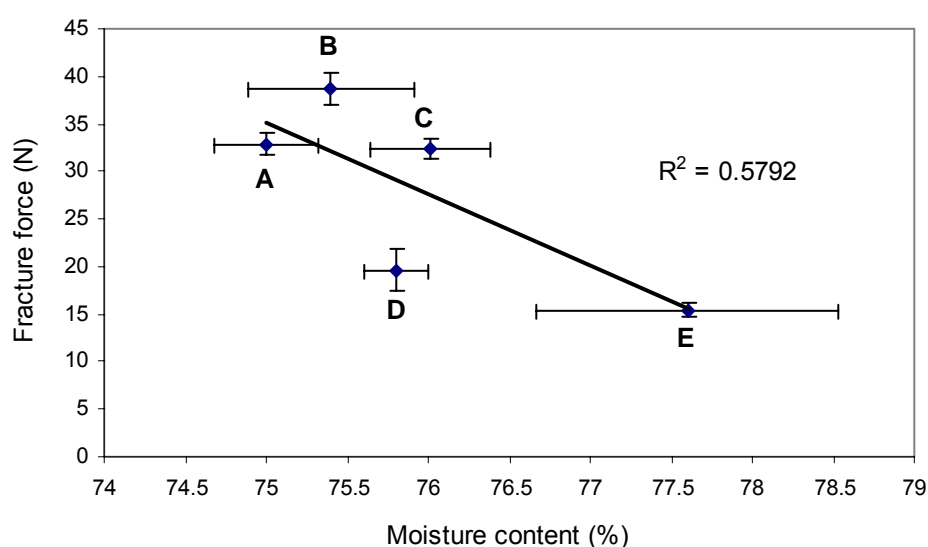


Figure 6.8: The relationship of fracture force and moisture content at the before boiling treatment. A, B, D and E are 1, 2, 15 and 30 mM glutaraldehyde, respectively. C is a control. Values are the means of five replicate measurements. Error bars represent standard error of the means.

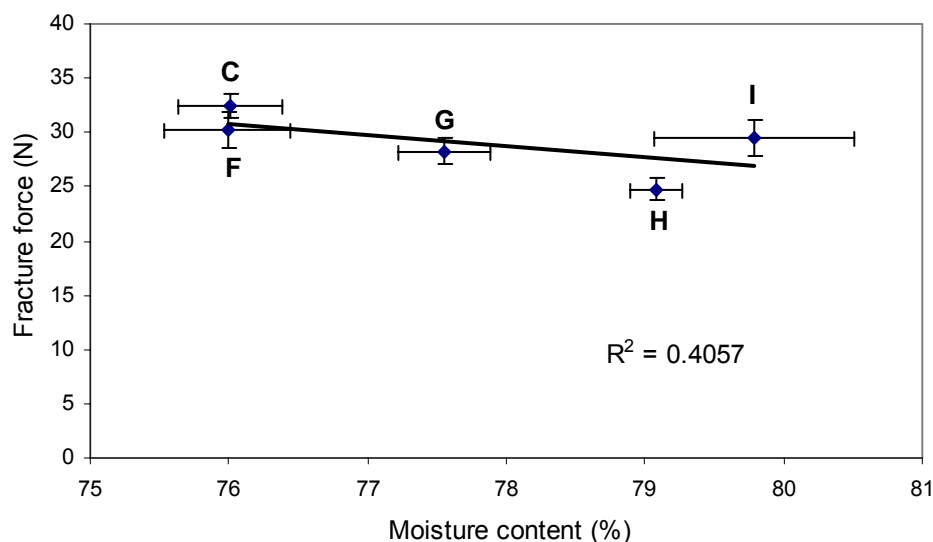


Figure 6.9: The relationship of fracture force and moisture content at the after boiling treatment. F, G, H and I are 1, 2, 15 and 30 mM glutaraldehyde, respectively. C is a control. Values are the means of five replicate measurements. Error bars represent standard error of the means.

6.4 Effect of TGA on textural properties of tofu

The commercial preparation of TGA consisted of only 20% enzyme, and low doses have not been reported to change the properties of foods. In earlier studies on other protein-based food, a high TGA concentration was needed to produce desirable effects on food products (Gerrard et al. 1996, Rasiah 2002). Based on this work, the TGA concentration was set at 1000 and 5000 ppm for this work, as used in Chapter 4. Two concentrations were used in order to compare the fracture force as well as mass balance.

6.4.1 Physical properties and protein content

Moisture content and density: The moisture content of soymilk, tofu and whey, and the density of tofu following TGA treatment are presented in Appendix 5. The moisture content of soymilk and whey in all treatments were constant at 90% and 97%, respectively. For tofu sample, the moisture content and density were in a range of 76-78% and 1.02-1.07 g/cm³, respectively.

The volume, weight and total water content: The volume, weight and total water content in the total input and output of all treatments are presented in Appendices 6, 7 and 8. The volume, weight and water content at total output were significantly reduced ($P < 0.05$) by 3-7%, 10-13% and 11-14%, respectively, indicating that these reductions were due to the loss of water.

The protein content: The protein content in the input and output are presented in Table 6.7. No significant difference ($P > 0.05$) was found between the total input and total output, suggesting that the total proteins were preserved during the experiment. However, the protein was partitioned between tofu and whey at 94% and 6%, respectively, for all treatments.

6.4.2 SDS-PAGE profiles

i) Treatment before boiling: The electrophoretic patterns of the proteins of soybean, soymilk, tofu and whey for treatment before boiling are presented in Figure 6.10. The electrophoretic profiles of soymilk treated with 1000 and 5000 ppm TGA did not show any protein cross-linking. The intensities of all protein bands remained similar to the control. A similar effect was also observed on the formation of aggregated proteins that smeared on the top the gel.

In the corresponding tofu samples (Figure 6.10), the electrophoretic profiles also showed no significant change in the intensities of protein subunits. However, there was a slight reduction of intensity of cross-linked protein observed on the top of the gel using 5000 ppm TGA (Lane 3) perhaps suggesting that larger aggregates are formed that could not enter the gel.

Table 6.7: The total protein content input and output in experiments with transglutaminase.

Treatment	Total input	Output		Total output	Total input versus Total output
	Soymilk (g)	Tofu (g)	Whey (g)	Tofu + Whey (g)	
1. Control	38.83 ± 0.72	37.12 ± 0.44	2.42 ± 0.05	39.54 ± 0.44	ns
2. 1000 ppm TGA-B	39.63 ± 0.24	37.29 ± 0.27	2.37 ± 0.05	39.66 ± 0.28	ns
3. 5000 ppm TGA-B	39.34 ± 0.51	36.30 ± 0.22	2.32 ± 0.05	38.62 ± 0.17	ns
4. 1000 ppm TGA-A	37.92 ± 1.07	37.53 ± 1.03	2.33 ± 0.03	39.86 ± 0.35	ns
5. 5000 ppm TGA-A	39.53 ± 1.48	36.65 ± 1.09	2.39 ± 0.07	39.04 ± 1.08	ns

Values are the means ± standard error of the means of five replicate measurements. ns = not significantly different ($P > 0.05$). TGA-B = Added TGA before boiling. TGA-A = Added TGA after boiling.

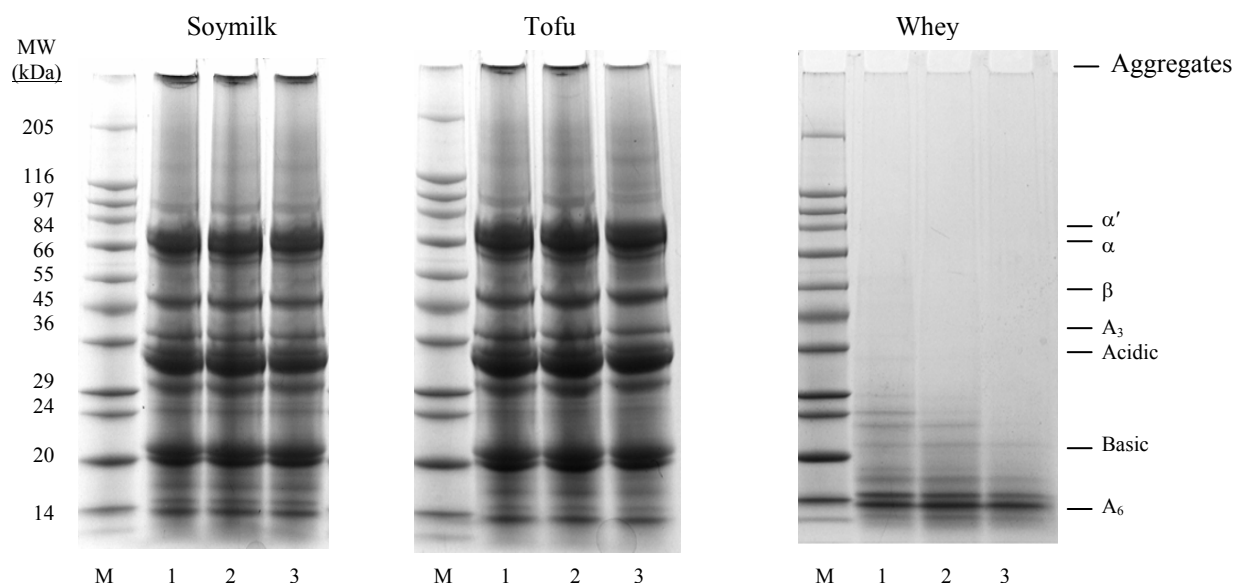


Figure 6.10: SDS-PAGE profile of proteins in soymilk, tofu and whey under reducing condition in the treatment before boiling. M = marker; 1 = control; 2 and 3 are 1000 and 5000 ppm TGA; The electrophoretic profiles are representative of duplicate gels.

In the corresponding whey samples, lower molecular weight protein bands were observed in a range of 14-24 kDa. The protein bands around 14 kDa were more intense than other bands. As the concentration increased from 1000 to 5000 ppm, a number of bands start to disappear, suggesting that lower molecular weight proteins were aggregated in the tofu.

Densitometric analysis: The electrophoretic profiles of Figure 6.10 were analysed densitometrically. The relative concentration of protein is presented in Table 6.8. In the soymilk, the composition of cross-linked proteins remained unchanged as the TGA concentration was increased from 1000 to 5000 ppm. In the corresponding tofu, the relative concentration of aggregated proteins at 1000 ppm TGA was unchanged, but when the TGA concentration was increased to 5000 ppm, the relative concentration of aggregated proteins was significantly reduced to 57%, perhaps suggesting that larger aggregates were formed that were insoluble in the extracting buffer.

Table 6.8: The changes of protein concentration in the cross-linked proteins and subunits in the TGA treatment added before soymilk boiling.

Aggregates and subunits	Relative protein concentration (% of control)			
	Soymilk		Tofu	
	1000 ppm	5000 ppm	1000 ppm	5000 ppm
Aggregates	100 ± 2	96 ± 1	95 ± 3	57 ± 4
$\alpha' + \alpha$	95 ± 9	85 ± 16	95 ± 5	97 ± 1
β	92 ± 8	84 ± 14	92 ± 9	95 ± 15
A ₃	86 ± 12	72 ± 21	93 ± 2	83 ± 3
A	91 ± 5	79 ± 13	95 ± 4	100 ± 1
B	88 ± 1	79 ± 16	95 ± 4	98 ± 11
A ₆	89 ± 5	69 ± 26	81 ± 4	93 ± 18

Values are the means ± standard error of the means of duplicate measurements. The percentage is based on the control soymilk and tofu respectively. Acidic comprised of A₁, A₂, A₄ and A₅ subunits. Basic comprised of B₁₋₄ subunits.

For the soymilk, the relative concentration of protein subunits was slightly reduced, ranging from 86 to 95% for 1000 ppm TGA, and from 69 to 85% for 5000 ppm TGA. In the corresponding tofu, the relative concentrations of subunits were also slightly reduced, falling in the range of 81-95% for 1000 ppm TGA, and 83-99% for 5000 ppm TGA. The reduction of relative concentration of protein subunits was not significantly different to the corresponding soymilks.

With the whey samples, as the TGA concentration increased, the relative concentration of proteins decreased to 77% of control at 1000 ppm, and 52% at 5000 ppm, indicating that these lower molecular weight proteins were aggregated in the tofu network.

ii) Treatment after boiling: The electrophoretic patterns of the proteins of soymilk, tofu and whey for treatment after boiling are presented in Figure 6.11. Since the soymilk was not treated with TGA, the electrophoretic patterns are similar. In the TGA-treated tofu,

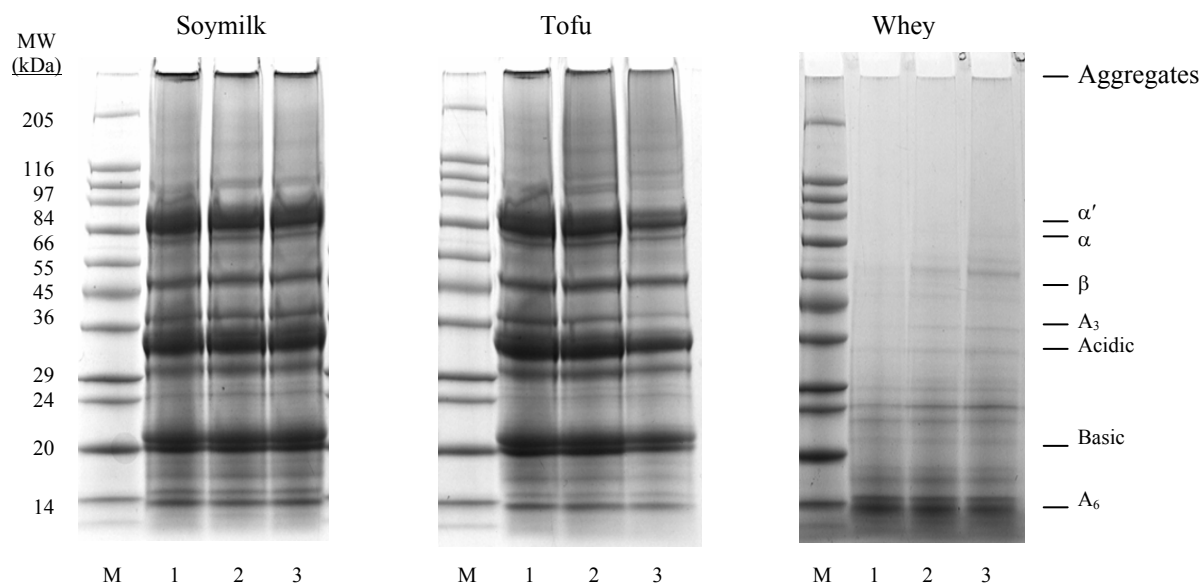


Figure 6.11: SDS-PAGE profile of proteins in soymilk, tofu and whey under reducing condition in the treatment after boiling. M = marker; 1 = control; 2 and 3 are 1000 and 5000 ppm TGA; Soymilk was not treated with TGA. The electrophoretic profiles are representative of duplicate gels.

the protein subunits of 1000 ppm TGA-treated tofu did not cross-link, but at 5000 ppm TGA, the intensities of the subunits bands decreased slightly, suggesting a small proportion of proteins were cross-linked. The intensity of the aggregated proteins smeared at the top of the gel, was also slightly decreased. These results suggest that the catalytic activity of TGA was slightly higher when added after boiling the soymilk than when added before boiling, consistent with greater substrate availability in the denatured protein. The cross-linking reaction appeared to favour the α' , α and A_3 subunits.

Densitometric analysis of the electrophoretic profiles of tofu is presented in Table 6.9. The protein composition of cross-linked protein observed on tofu confirmed a slight reduction to 91% for 1000 ppm TGA, and 80% for 5000 ppm TGA. The subunits of the 1000 ppm TGA-treated tofu showed no cross-linking had occurred. However, when the TGA concentration was increased to 5000 ppm, the relative concentrations of these subunits were reduced slightly to 68-98% of the control.

In the corresponding whey, the electrophoretic profile was in contrast to those obtained for treatment before boiling. As the TGA concentration was increased, the number of low molecular weight proteins present in the whey increased, although the protein bands around 14 kDa were still dominant. These results suggest that TGA had hydrolysed glutamine residues to glutamate residues of soy proteins which increased the solubility and allowed them to be extracted and appear on the gel (Cortez et al. 2004, Flanagan and Fitzgerald 2002).

Table 6.9: Relative concentrations of aggregated proteins and proteins subunits of tofu for TGA treatment following boiling of the soymilk.

Cross-linked and subunits	Relative protein concentration in tofu (% of control)	
	1000 ppm TGA	5000 ppm TGA
Aggregates	91 ± 6	80 ± 7
$\alpha' + \alpha$	92 ± 3	74 ± 5
β	100 ± 1	88 ± 9
A ₃	86 ± 11	68 ± 17
A	100 ± 1	79 ± 8
B	100 ± 1	90 ± 5
A ₆	93 ± 8	85 ± 11

Values are the means ± standard error of the means of duplicate measurements. The percentage is based on the control tofu respectively.

In the corresponding whey samples, densitometric analysis confirmed that the protein composition had increased to 104% and 130% for 1000 and 5000 ppm TGA respectively, as compared to the control.

6.5 The textural properties of TGA-treated tofu

Fracture force profile: A typical force-deformation curve representing all treatments of TGA-treated tofu is presented in Figure 6.12. The force increased with

compression, and concomitantly, the modulus also increased, before decreasing. The maximum peaks of force and modulus at which the tofu was fractured are denoted as **a** and **b**, respectively.

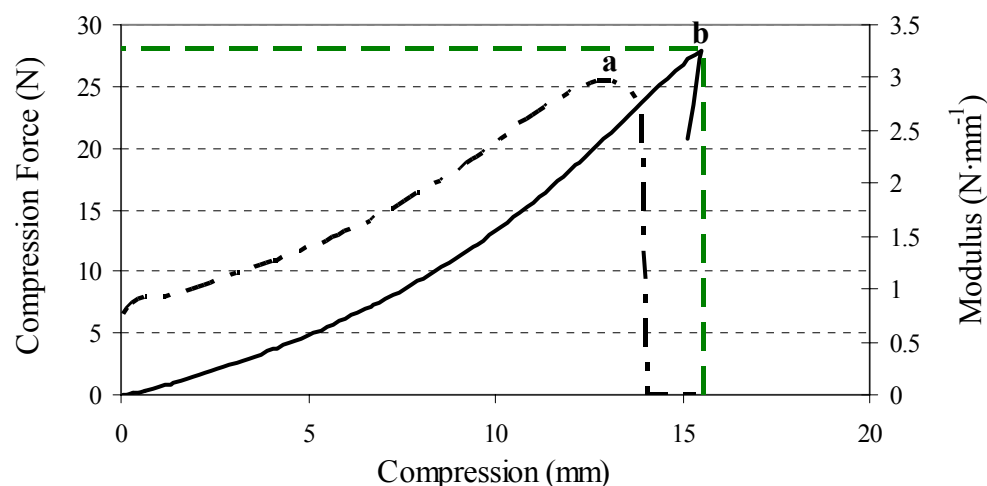


Figure 6. 12: A typical force-deformation compression curve of TGA-treated tofu. Symbols **a** and **b** represent the maximum compression modulus and fracture point at maximum compression, respectively. — Force; — — Maximum compression; — - — Modulus. The profile was adapted from a treatment of 1000 ppm TGA added after soymilk boiling.

Fracture distance: The fracture distance was in a range of 14.90-15.66 mm as presented in Table 6.10. The corresponding fracture forces are shown in Figure 6.13.

Table 6.10: The fracture distance of TGA-treated tofu samples.

TGA (ppm)	Added before boiling	Added after boiling
0	14.41 ± 0.42	14.41 ± 0.42
1000	14.90 ± 0.42	15.44 ± 0.16
5000	15.56 ± 0.02	15.66 ± 0.04

Values are the means ± standard error of the means of five replicate measurements.

Fracture force: The fracture force of TGA-treated tofu is presented in Figure 6.13. Compared to the control, the fracture force at both TGA concentrations, whether added before or after soymilk boiling, increased significantly ($P < 0.05$). Further, with the increase in TGA concentration both for treatment before and after boiling, the fracture force significantly increased ($P < 0.05$). However, the fracture force for the tofu treated after boiling was lower than that for those treated before boiling.

The lower fracture force for the sample treated after boiling is attributed to the TGA reaction period in the soymilk. With the treatment after boiling, TGA was allowed to react for 8 min at 50°C, during which time the gelation was also taking place. After the soymilk had formed a gel, the accessibility of TGA to the substrates was perhaps limited. On the other hand, with treatment before boiling, the reaction period for TGA at 50°C was also fixed at 8 min, but the accessibility of TGA to the substrates was not hindered by gelation. Therefore, TGA could continue to catalyse the proteins in the soymilk until deactivated at 70°C (Motoki and Seguro 1998).

Regardless of which juncture TGA is added, the fracture force increased with increasing concentration, even though only a few cross-links were formed. This is in contrast to the results seen for glutaraldehyde cross-linking.

Compression modulus: To corroborate the fracture force profiles, the compression modulus was also measured as presented in Figure 6.14. The compression modulus followed the same pattern as the fracture force. The compression modulus of all TGA concentrations was significantly increased ($P < 0.05$) over the control. This confirmed that the stiffness and firmness of the tofu had increased (Huang et al. 2003, Visser 1991).

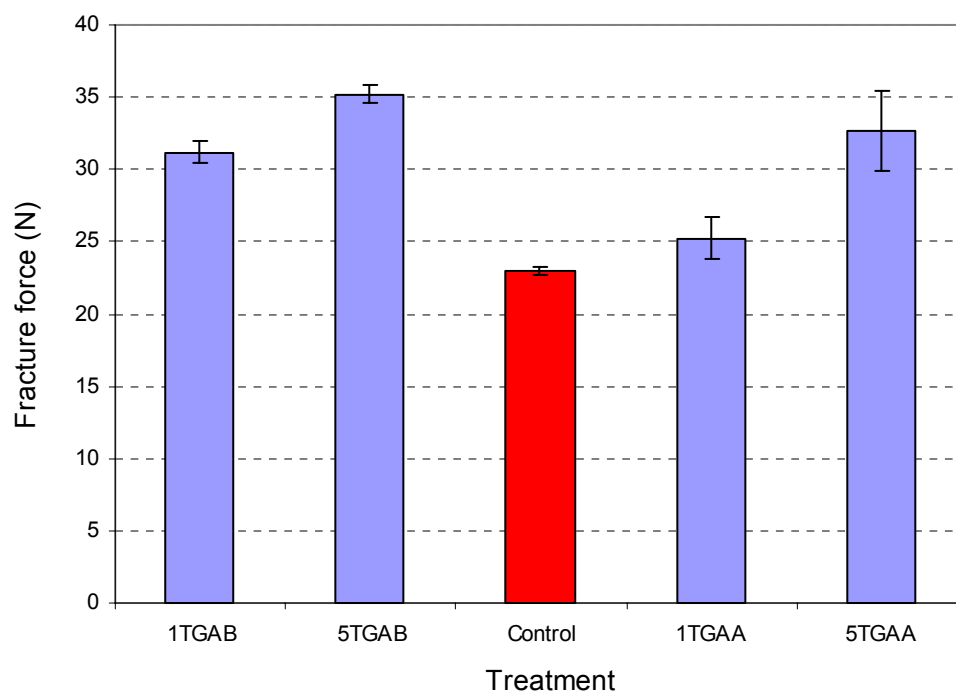


Figure 6.13: The fracture force in each treatment of TGA cross-linking. Values are the means of five replicate measurements. Error bars represent the standard errors of the means. 1TGAB and 5TGAB are 1000 and 5000 ppm TGA added before soymilk boiling. 1TGAA and 5TGAA are 1000 and 5000 ppm TGA added after soymilk boiling.

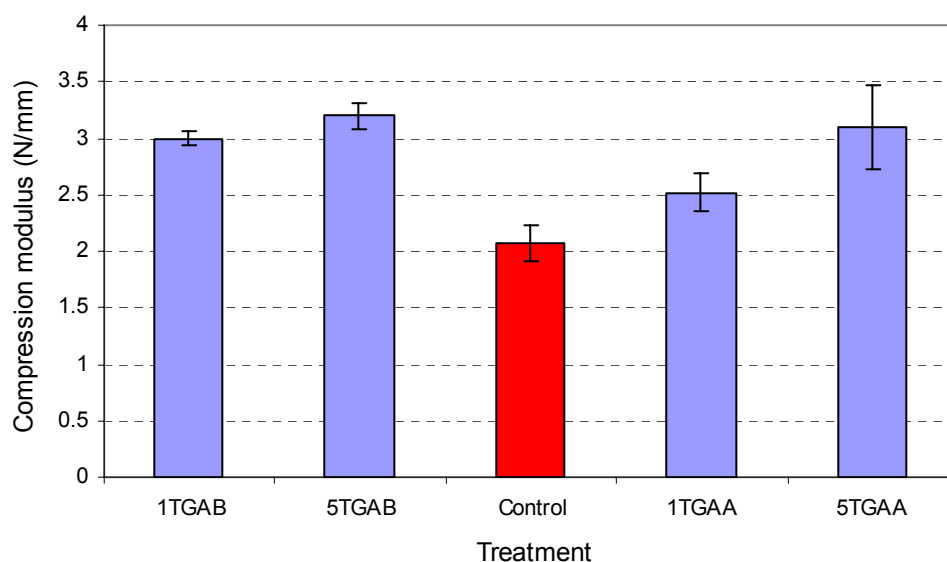


Figure 6.14: The compression modulus in each treatment of TGA cross-linking. Values are the means of five replicate measurements. Error bars represent the standard error of the means. 1TGAB and 5TGAB are 1000 and 5000 ppm TGA added before soymilk boiling. 1TGAA and 5TGAA are 1000 and 5000 ppm TGA added after soymilk boiling.

6.5.1 Relationship between fracture force and moisture content

The relationship between the fracture force and moisture content was also examined using the available data, including the control. The trends of treatment before and after boiling are presented in Figure 6.15 and 6.16. In the before boiling treatment, only a weak correlation was obtained. However, the general trend shows that as the moisture content concentration increased, the fracture force decreased.

In contrast, with the treatment after boiling, the fracture force increased when the moisture content increased, and had a strong relationship. Overall, it seems that the moisture content did not correlate to the firmness of tofu.

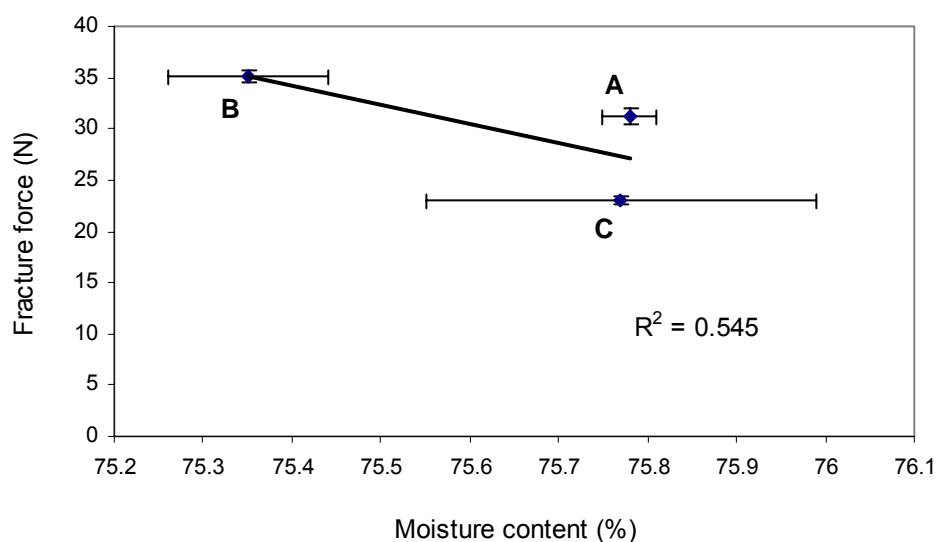


Figure 6.15: The relationship of fracture force and moisture content at the treatment before boiling. A and B is 1000 and 5000 ppm TGA. C is a control. Values are the means of five replicate measurements. Error bars represent standard error of the means.

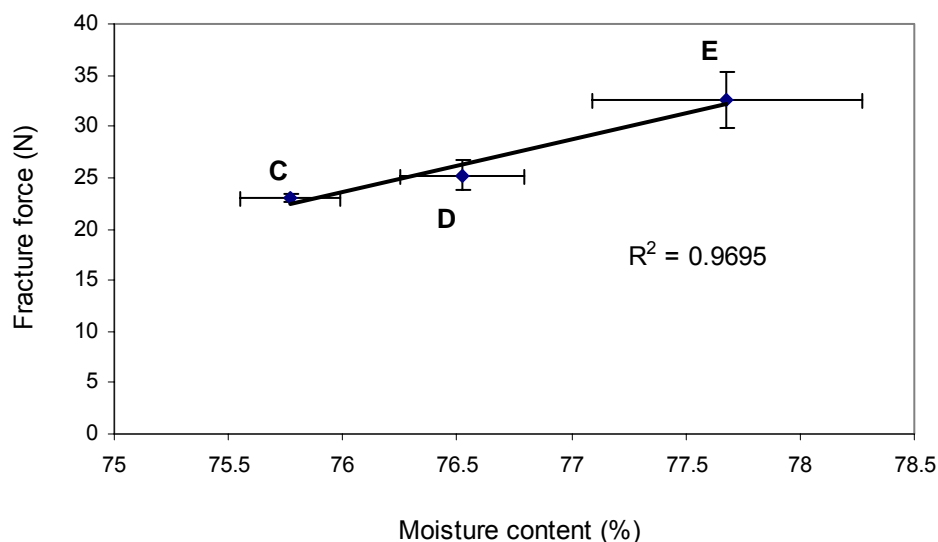


Figure 6.16: The relationship of fracture force and moisture content at the treatment after boiling. D and E is 1000 and 5000 ppm TGA. C is a control. Values are the means of five replicate measurements. Error bars represent standard error of the means.

6.5.2 Mechanism of TGA catalysis in soy proteins

In this study, only slight cross-linking was observed on the SDS-PAGE gel, in contrast to the *in vitro* incubation (Chapter 4), and yet the fracture force was increased. This indicates that another mechanism has led to strengthening of the protein-protein bonding.

Since water was abundant during tofu manufacture, it is likely that TGA promoted hydrolysis of glutamine residues of the protein to glutamate residues by one of two mechanisms: i) either the ϵ -(γ -glutamyl)lysyl cross-link is hydrolysed into glutamate and lysine, or ii) glutamyl residue is hydrolysed directly into glutamate, as shown in Figure 6.17 (Sharma et al. 2001, Yokoyama et al. 2004).

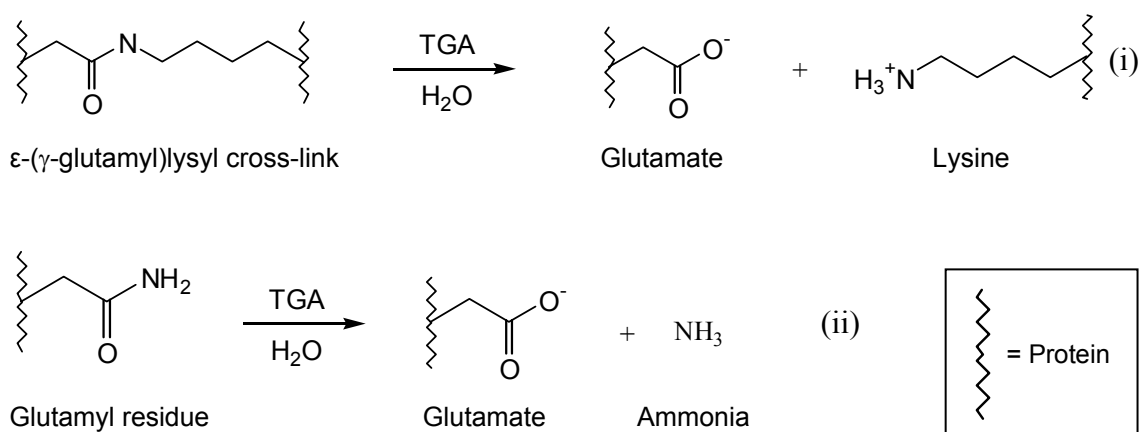


Figure 6.17: The hydrolysis of glutamyl residue of the protein by TGA.

Aggregates are the main structural blocks for tofu gelation (Doi 1993). Coagulating heat-denatured proteins by adding salt (Ca^{2+} ions) facilitates the transition of soymilk solution into gel. In this transition, the interaction occurred among aggregates was attributed by non-covalent bonds, which determined the texture of the gel (Ju and Kilara 1998). It is believed that surface properties of the aggregated proteins such as hydrophobicity or electrostatic interaction, have been changed which led to the formation of firm tofu. Thus an increase in the number of negatively charged residues in the denatured soy protein may account for a difference in tofu properties.

6.6 Conclusion

As evidenced by SDS-PAGE gels, cross-linking a small number of proteins using low concentrations of glutaraldehyde and added before soymilk boiling resulted in an increase in fracture force. However, the fracture force did not change when glutaraldehyde was added after soymilk boiling. In contrast, extensive cross-linking using higher concentrations of glutaraldehyde resulted in a lowering of the fracture force. This occurred whether the glutaraldehyde was added before or after soymilk boiling.

Following TGA treatment, only a small degree of cross-linking was observed on the SDS-PAGE gel, but the fracture force was increased. It is hypothesized that hydrolysis of glutamine residues to glutamate residues may have altered the aggregation properties of the soy protein. As a consequence, the fracture force increased. The lower fracture force in the treatment after boiling compared to the treatment before boiling was attributed to the shorter catalysis period for TGA in soymilk in the former case.

The fracture force did not correlate with the moisture content or degree of cross-linking, for either glutaraldehyde or TGA treatment. To further examine the reasons for the change in fracture force upon treatment with glutaraldehyde and transglutaminase, the microstructure of the samples was examined, as described in Chapter 7.

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CHAPTER 7

THE EFFECT OF GLUTARALDEHYDE AND TRANSGLUTAMINASE ON THE MICROSTRUCTURE OF TOFU

7.1 Background

In Chapter 6, glutaraldehyde and TGA were used to cross-link soy proteins and found to change the textural properties of tofu. These changes were hypothesised to correlate with modification to the microstructure of the three-dimensional tofu network. Scanning electron microscopy (SEM) has been previously employed to examine the surface structure of the food, and relate the structure to texture (Aguilera 2005, Khaláb et al. 1995, Wilkinson et al. 2000). Therefore, the main objective of this chapter was to correlate the microstructure of tofu to the textural properties. To accomplish this objective, SEM was used to capture high magnification images of control, glutaraldehyde- and TGA-treated tofu. SEM is a powerful technique for studying foods, and has previously been used to study soy protein gels (Hermansson and Buchheim 1981). Since tofu is a highly hydrated product, the preparation of dehydrated samples for SEM may result in the formation of preparation artifacts; however, replicated differences between treatments still suggest that the cross-linking treatments produce changes to structure that are reflected in the hydrated material.

7.2 Microstructure of control tofu

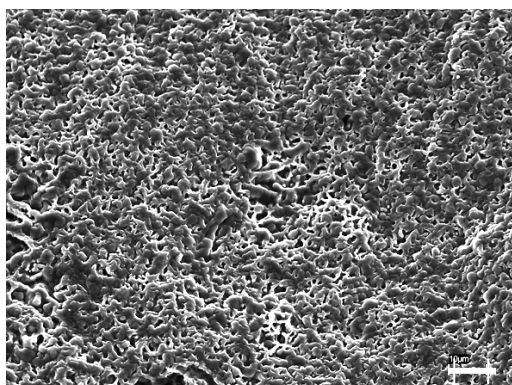
The changes in textural properties of tofu have been correlated with the changes of microstructure (Kao et al. 2003, Lee and Rha 1978, Meng and Ma 2002, Noh et al. 2005, Tsintsadze et al. 1978). Saio (1979) reported that the hardness of tofu depends on the density of the gel network and the size of protein granules. Different types of

coagulant employed also influenced the microstructure and texture of tofu (deMan et al. 1986).

In manufacturing glutaraldehyde-treated control tofu, soymilk was heated to 97°C to denature the soy proteins. Subsequently, this was cooled to 87°C, calcium sulfate was added and coagulation was allowed to proceed for 8 min. The tofu gel was then loaded into a mould and pressed with a 600 g load for 1 hr. The formed tofu was left at room temperature (20°C) overnight prior to fracture testing and SEM microstructure analysis. The preparation of TGA-treated control tofu proceeded as for glutaraldehyde control tofu, except coagulation was carried out at 50°C, corresponding to the maximum temperature at which TGA remains active, rather than 87°C.

The freeze-fracture method is described in section 9.6 (Chapter 9). The difference in observed microstructure is believed to be due to the difference in a coagulation regime, 87°C for glutaraldehyde-treated tofu and 50°C for TGA-treated tofu.

Glutaraldehyde control



TGA control

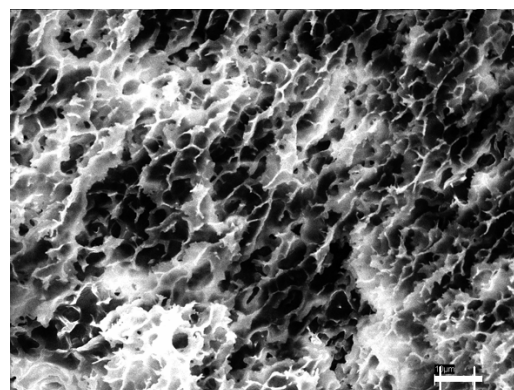


Figure 7.1: SEM micrographs of glutaraldehyde- and TGA-treated control tofus. The image is a representative of five replicate observations of five replicate control samples. The SEM images were captured at 2000x magnification.

The microstructure of glutaraldehyde-treated control tofu showed small pores distributed uniformly in a dense network. The network is well inter-connected. In contrast, the TGA-treated control tofu had larger pores, and consisted of a “flakey-type” network. It also showed open-wide pores with a loose network and diffuse structure

(Aluko and Yada 1999, Heertje 1993, Mohamad Ramlan et al. 2004). Foegeding, (2003) described that the fracture of a gel material (in this case soy proteins) and inter-molecular bondings are inter-related. The well inter-connected structure showed by the glutaraldehyde-treated control tofu presumably had strong protein-protein interaction, which contributed to a higher fracture force. On the other hand, the loose network was probably a result of weaker protein-protein interaction, causing a decrease in fracture force.

7.3 Gelation mechanism

A general mechanism of tofu gelation is described in Figure 1.11 in Chapter 1. The firmness of tofu is mainly governed by covalent and hydrogen bonds, and also hydrophobic and electrostatic interactions (Dickinson 1997, Dickinson and Yamamoto 1996). After microscopic observation, a number of models for the mechanism of gelation during tofu manufacture have been proposed (Kao et al. 2003, Kohyama et al. 1995, Nakamura et al. 1984, Utsumi et al. 1984). Protein aggregation is initiated by the formation of strands during heating, and the linkages of these strands form “ring-like” structure (Hermansson and Buchheim 1981). For gels of globular protein, strands have been observed as linear filaments, that look like “strings of beads” (Doi 1993, Hermansson 1986). Thus, these are the building blocks of the tofu structure. At the molecular level, upon denaturation, the filamentous strands are formed mainly by the interaction of β -subunits of β -conglycinin and the B-subunits of glycinin. The α' and α subunits of β -conglycinin and A-subunits of glycinin remain as uncomplexed monomers (Utsumi et al. 1984).

In a fine-stranded gel network, proteins are attached to each other, typically as a “string of beads”, whilst in a coarse gel, the network is due to random aggregation that forms “clumps” with a thick-stranded network (Renkema 2001). Wilcox and Swaisgood (2002) described that the formation of particulate gels might contributed to the brittleness of the gel.

In a fine-stranded network, proteins were reported to link by disulfide bonds, as well as electrostatic association between the dissociated β -conglycinin subunits and B-subunits of glycinin (Hermansson 1986). An increase of disulfide bonds in glycinin was found to increase the breaking force of tofu (Saio et al. 1971).

The proposed gelation mechanism of glutaraldehyde- and TGA-treated control tofu is schematically illustrated in Figure 7.2. In this figure, the pore size of glutaraldehyde-treated control tofu is smaller than the TGA-treated control tofu. In their corresponding whey fractions, TGA control tofu contained more lower molecular weight proteins than glutaraldehyde control tofu, as seen in the SDS-PAGE profiles (Figure 6.2 and 6.12).

7.4 Microstructures of glutaraldehyde-treated tofu

The microstructures of glutaraldehyde-treated tofu are divided into two concentrations: i) lower concentrations (1 and 2 mM), and ii) higher concentrations (15 and 30 mM). The moisture content was 75% and in a range of 76 – 78% for lower and higher concentration, respectively (Appendix 1). In each group, the microstructures are compared and related to their fracture force.

7.4.1 Microstructure at low concentrations

The microstructures at low concentrations and their respective fracture forces are presented in Figure 7.3. Their respective SDS-PAGE profiles were shown in Chapter 6 (Figures 6.2 and 6.4). The moisture content was 75% and in a range of 76 – 78% for before and after boiling treatment, respectively (Appendix 1).

Before boiling treatment: The microstructures of 1 and 2 mM glutaraldehyde-treated are shown Figures 7.3A and B. The microstructure at 1 mM glutaraldehyde tofu shows a honey-comb like structure with relatively large pore sizes and shows a thick-stranded network. The network is mostly regular and the pores are uniformly distributed.

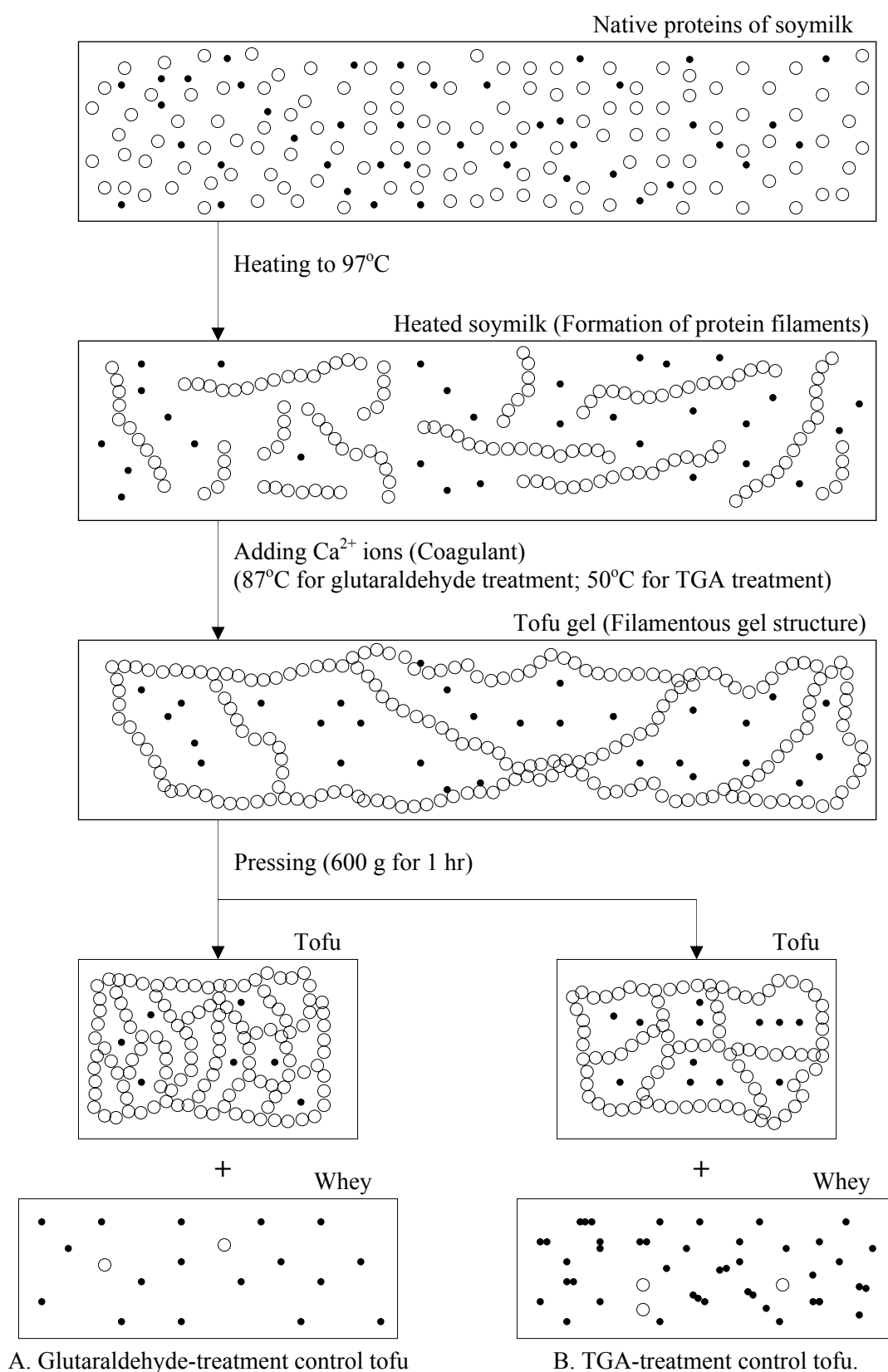
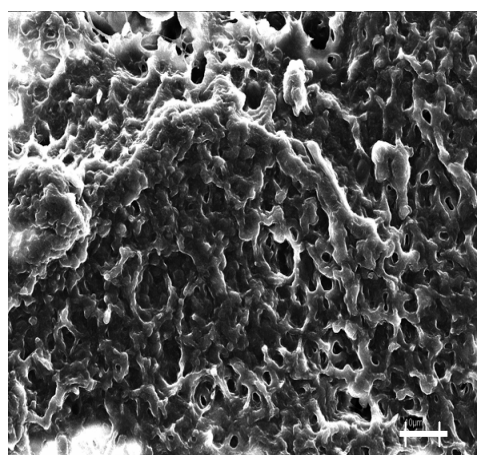
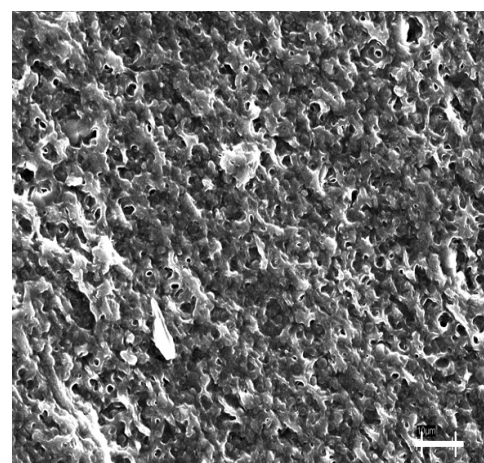


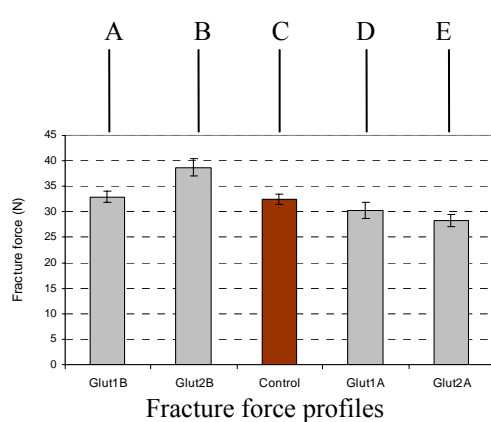
Figure 7.2: Gelation mechanism of control tofu. A = glutaraldehyde treatment. B = TGA treatment. (\circ) and (\bullet) represent high and low molecular weight proteins, respectively. Adapted from Kao et al. (2003).



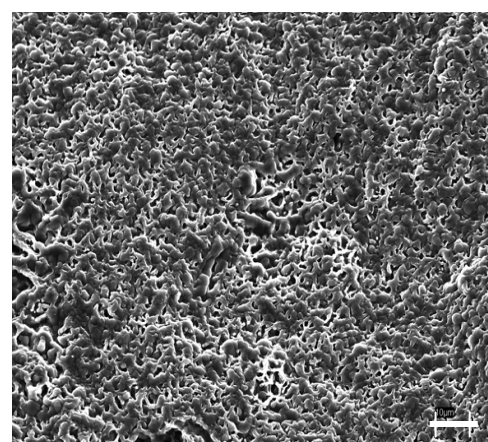
A: 1 mM glutaraldehyde before



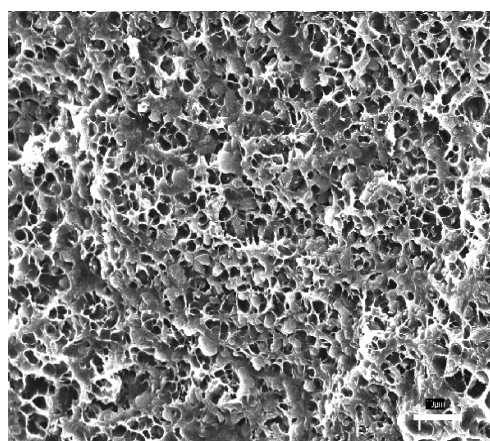
B: 2 mM glutaraldehyde before



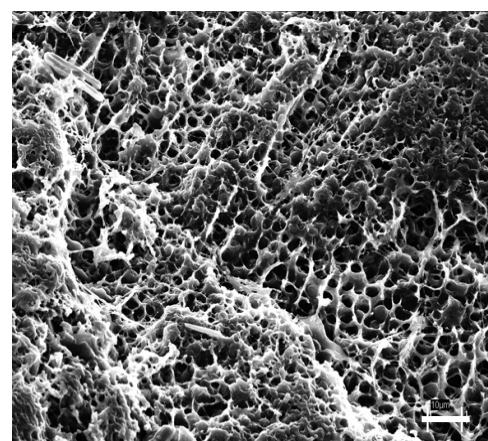
Fracture force profiles



C: Control



D: 1 mM glutaraldehyde after



E: 2 mM glutaraldehyde after

Figure 7.3: The SEM micrographs of lower level of glutaraldehyde-treated tofu corresponding to their respective fracture force. A and B represent micrographs of 1 and 2 mM glutaraldehyde added before soymilk boiling. C represents control. D and E represent micrographs of 1 and 2 mM glutaraldehyde added after soymilk boiling. The image are representative of five replicate experiments. The SEM images were captured at 2000x magnification.

When the concentration of glutaraldehyde was increased to 2 mM, the microstructure comprised a smaller pore size with a more dense and compact network. The structure was again uniform. Since the pore size of the 2 mM glutaraldehyde-treated tofu was relatively small, with a denser and more compact network, the resulting fracture force was significantly higher than the fracture force of 1 mM glutaraldehyde.

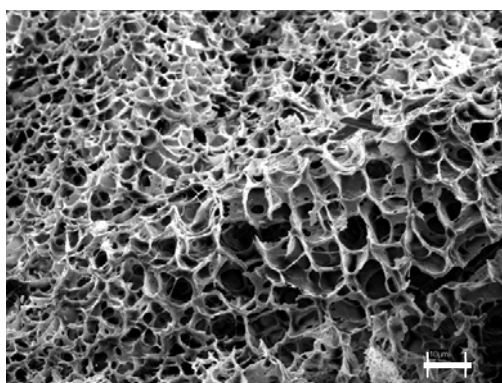
In the SDS-PAGE profile (Figure 6.2–Before boiling), a small number of proteins were cross-linked. The amount of cross-linked protein increased slightly by using 2 mM glutaraldehyde.

After boiling treatment: The microstructures are compared as presented in Figure 7.3D and E. The microstructures at both concentrations show honey-comb like pores with a continuous network and regular strands. The distribution of pores was uniform. However, in terms of porosity, the larger pores in the 2 mM glutaraldehyde-treated tofu are more than those of 1 mM. The resulting fracture forces of 1 and 2 mM glutaraldehyde treated tofu were lower than the control.

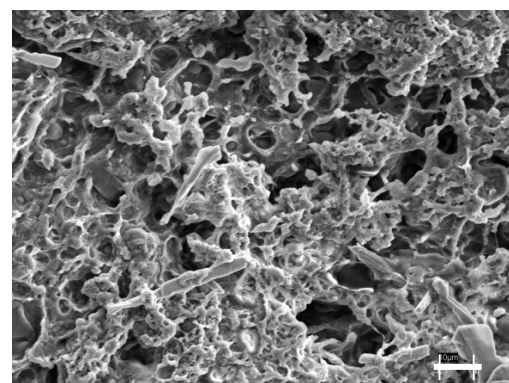
The SDS-PAGE profile (Figure 6.4–After boiling) showed that the quantity of cross-linked protein increased with increasing concentration of glutaraldehyde, and was higher than that seen with treatment before boiling, particularly in the β -conglycinin fraction. These results suggest that when the cross-linking increased, the fracture force decreased. However, the resulting microstructures are hard to rationalize in terms of the cross-linking patterns alone and suggest that other factors may be at play.

7.4.2 Microstructure at high concentrations

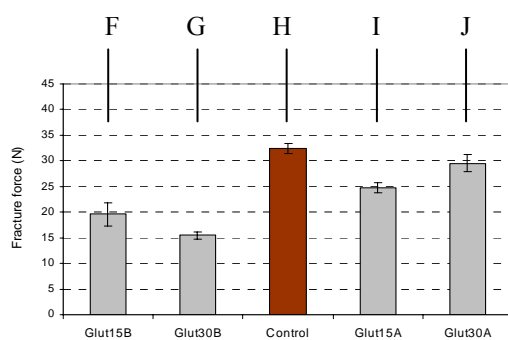
The microstructures and their respective fracture forces are presented in Figure 7.4. The moisture content was in a range of 76 – 78% and 79 – 80% for before and after boiling treatment, respectively (Appendix 1). Their respective SDS-PAGE profiles were presented in Chapter 6 (Figure 6.3 and 6.5).



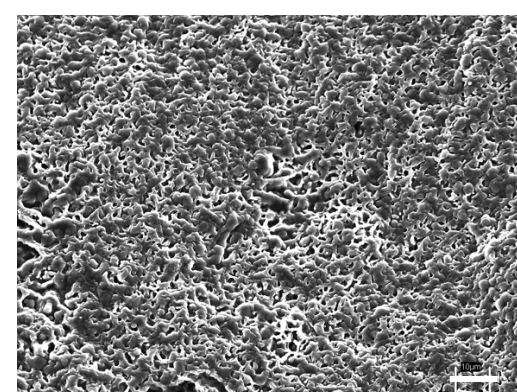
F: 15 mM glutaraldehyde before



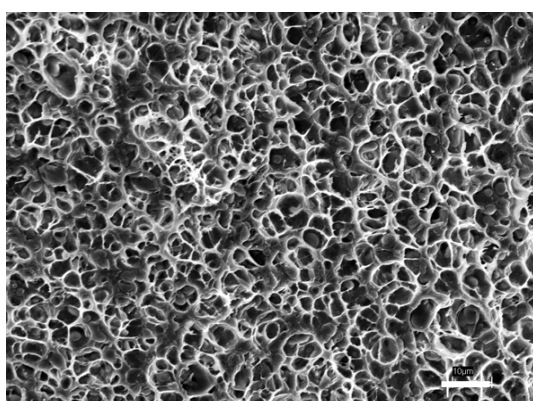
G: 30 mM glutaraldehyde before



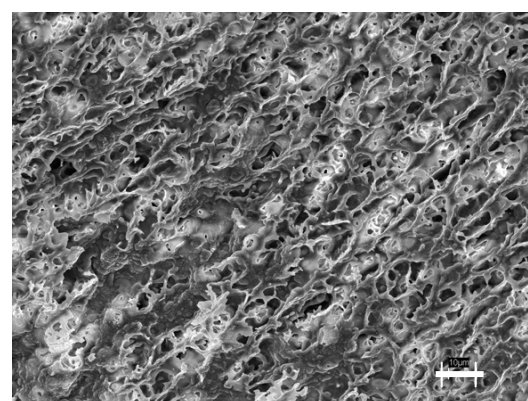
Fracture force profiles



H: Control



I: 15 mM glutaraldehyde after



J: 30 mM glutaraldehyde after

Figure 7.4: The SEM micrographs of higher concentrations glutaraldehyde-treated tofu corresponding to the respective fracture force. F and G represent micrographs of 15 and 30 mM glutaraldehyde added before soymilk boiling. H represents control. I and J represent micrographs of 15 and 30 mM glutaraldehyde added after soymilk boiling. The image is a representative of five replicate experiments. The SEM images were captured at 2000x magnification.

Treatment before boiling: The microstructure of 15 mM glutaraldehyde (Figure 7.4F) shows a thin-stranded network forming honey-comb like pores. However, the pore size was much bigger than those formed at low concentrations of glutaraldehyde. At 30 mM glutaraldehyde (Figure 7.4G), the microstructure showed larger aggregates and disintegration of the network between the aggregates, resulting in a lower fracture force. Physically, the tofu became brittle.

After boiling treatment: The microstructure of 15 mM glutaraldehyde-treated tofu (Figure 7.4I) shows a honey-comb like structure with quite uniform pore sizes. The pores are surrounded by a thin-stranded networks to form a “sponge-like” surface. In contrast, the 30 mM glutaraldehyde-treated tofu (Figure 7.4J) had fewer pores. The structure was mostly built up of a thick-stranded network with less porosity. A thicker stranded network resists bending and requires higher force to fracture (Renkema 2004).

The SDS-PAGE profiles (Figures 6.3 and 6.5) of treatment before and after boiling show that the proteins were completely cross-linked to form larger aggregates. Only a small proportion of B subunits were not cross-linked when 15 mM glutaraldehyde was added before boiling.

7.4.3 Comparing low and high concentrations

In general, the microstructure of the higher concentrations of glutaraldehyde showed larger pore sizes (Figure 7.4F, G, I and J) than those of lower concentrations (Figure 7.3A, B, D and E). Larger pores resulted in lowering fracture force, whilst compact structure of lower concentration led to increasing fracture force.

In the after boiling treatment, when proteins unfolded as a result of heat-denaturation, more cross-linking was observed on the SDS-PAGE gel. Interestingly, both lower and higher concentrations of glutaraldehyde caused a decrease in fracture force, slightly lower than the control. These results suggest that cross-linkings can result in the lowering of fracture force.

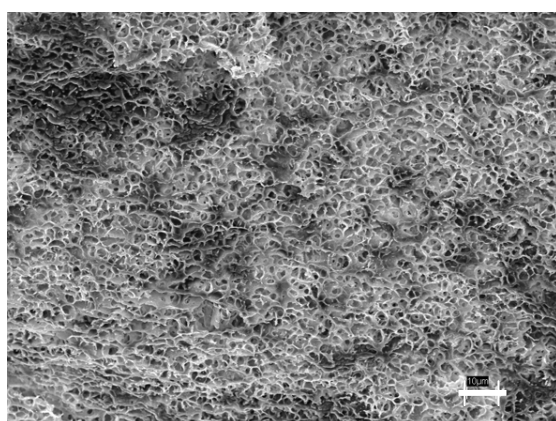
7.4.4 Gelation mechanism of glutaraldehyde treatment

These results suggest that the gelation mechanism in the glutaraldehyde-treated tofu did not depend on the cross-linking alone, but other reactions are also taking place such as non-enzymatic glycation between soy proteins and glutaraldehyde. Since the manufacture of tofu employed considerably high temperature during soymilk boiling (97°C), the addition of glutaraldehyde can promote non-enzymatic glycation through various series of complex pathways. In the advance stage of glycation, the brown polymers and co-polymers are produced (Hodge 1953). In this experiment, brown colour was seen as a result of using 15 and 30 mM glutaraldehyde, when added prior to or after soymilk boiling.

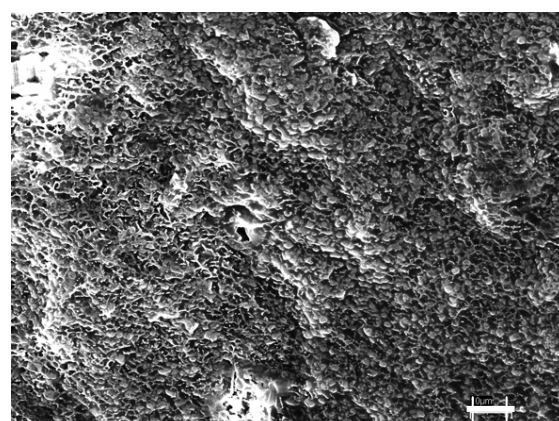
Glycated proteins have a different isoelectric point to the native form (Peters and Richards 1977). The functional properties of proteins in food are dependent on the molecular structure of proteins (Kinsella 1979). By changing the chemical properties of soy proteins, the functional properties are expected to change. Therefore, it is likely that protein glycation influenced the properties of tofu as well as the introduction of cross-links.

7.5 Microstructure of TGA-treated tofu

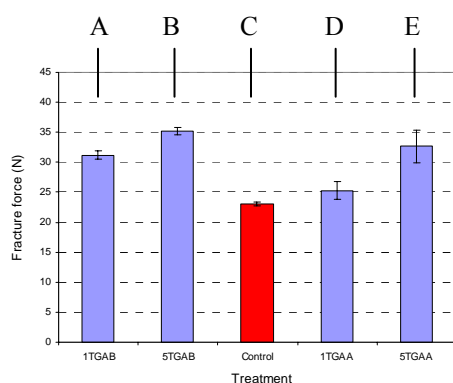
The microstructures of tofu treated with 1000 ppm and 5000 ppm TGA before and after boiling are shown along with their fracture forces in Figure 7.5. The corresponding SDS-PAGE profiles of tofu following TGA treatment was described in Chapter 6 (Figures 6.10 and 6.11).



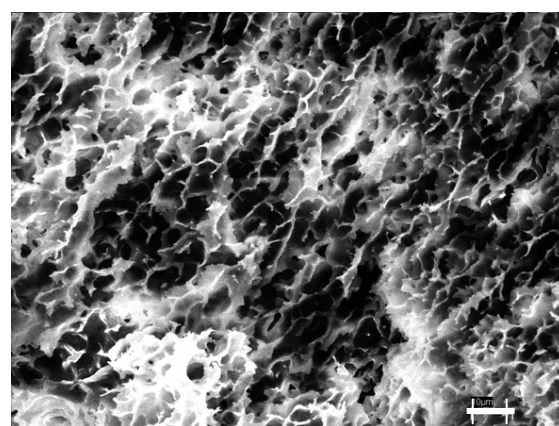
A: 1000 ppm TGA before



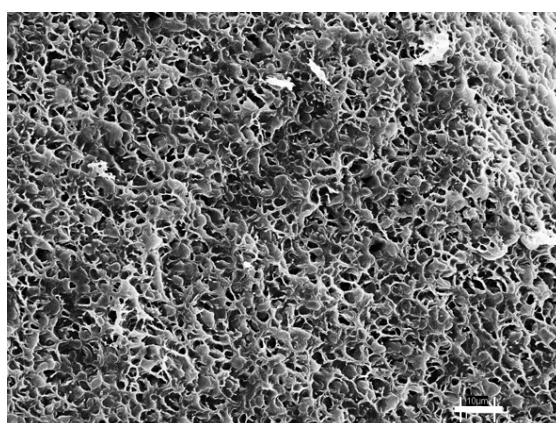
B: 5000 ppm TGA before



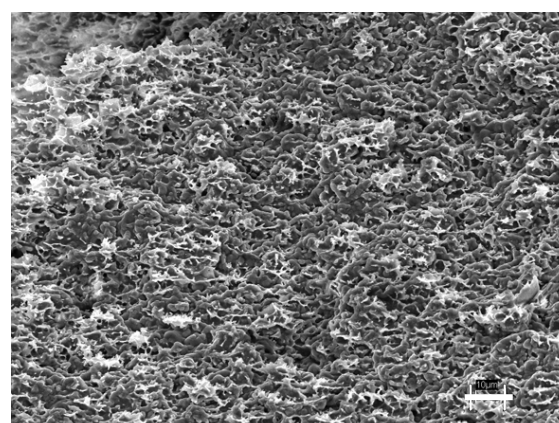
Fracture force profiles



C: Control



D: 1000 ppm TGA after



E: 5000 ppm TGA after

Figure 7.5: The SEM micrographs of TGA-treated tofu and the respective fracture force. A and B represents microstructure of 1000 and 5000 ppm TGA added before soymilk boiling. C represents the control. D and E represents microstructure of 1000 and 5000 ppm TGA added after soymilk boiling. The images are representative of five observations of replicate experiments. The SEM images were captured at 2000x magnification.

7.5.1 Microstructure of tofu treated before and after boiling

Treatment before boiling: Using 1000 ppm TGA (Figure 7.5A), the microstructure showed a fine-stranded network with smaller honey combs. The structure is uniformly distributed. However, when the concentration of TGA was increased to 5000 ppm (Figure 7.5B), the microstructure did not show honey combs and the aggregates seemed more rigid. Thus, with transformation from a fine-stranded network to a rigid structure, the fracture force of tofu increased.

The fracture force of 1000 ppm TGA-treated tofu was similar to the glutaraldehyde-treated control tofu. Their respective microstructures were also similar, suggesting that a similar microstructure resulted in similar fracture force.

Treatment after boiling: A similar trend in microstructure was observed with treatment after boiling. With 1000 ppm TGA (Figure 7.5D), the microstructure showed a uniform and continuously associated fine-stranded network with small pore sizes, showing a “sponge-like” structure. This resulted in the lowest fracture force among all TGA treatments. When the concentration of TGA was increased to 5000 ppm (Figure 7.11E), a fine-stranded network was also formed with less apparent honey combs. This structure resulted in increasing the fracture force.

In the corresponding SDS-PAGE (Figures 6.10 and 6.11), only a small degree of cross-linking occurred in tofu treated either added before or after boiling, suggesting that either a low level of cross-linking was capable of change the structure and texture of tofu, or that TGA li promoted hydrolysis of glutamine residue to glutamate that changed the isoelectric point of the soy proteins. As a consequence, by increasing the numbers of negative charges in the proteins, the solubility and emulsification of soy proteins improves and subsequently changes the functional properties of soy proteins (Hamada 1992).

Role of TGA on microstructure: Compared to the control, TGA treatment generally promoted the formation of fine-stranded networks and gave a more homogeneous structure with a higher breaking strength (Liu et al. 2004). Coarsening the network structure will decrease in gel rigidity (Verheul and Roefs 1998) as observed in

glutaraldehyde treatment. Gelation induced by Ca^{2+} ions showed a microstructure consisting of strands and contributed to higher rigidity and elasticity of gel, compared to thermally-induced gel (Chanyongvorakul et al. 1995). In other protein systems, a higher strength of TGA-induced gel was due to well-organised protein networks with smaller pores in the products (Faergemand and Qvist 1997, Farnsworth et al. 2005, Lorenzen et al. 2002, Schorsch et al. 2000).

7.5.2 Gelation mechanism of TGA treatment

A general mechanism for tofu gelation following treatment with TGA before and after soymilk boiling is schematically presented in Figures 7.6 and 7.7. Given the SDS-PAGE profile of the treatment before soymilk boiling (Figure 6.10) suggested little cross-linking. The process may have led to hydrolysis of glutamine residues, followed by the formation of protein filaments and gelation by coagulant. After compression, 5000 ppm TGA produced a tofu network with smaller pores than those in 1000 ppm TGA treated tofu.

Meanwhile, in the treatment after boiling (SDS-PAGE profiles of Figure 6.11), the addition of TGA concurrently with calcium sulfate resulted in two competing reactions. Calcium sulfate coagulated the protein filaments while TGA is hypothesised to have catalysed deamidation of these filaments. The hydrolysis produced glutamic acid residues on these filaments. The two concurrent reactions produced fine-stranded networks. 5000 ppm TGA-treated tofu had relatively smaller pores than 1000 ppm TGA-treated tofu.

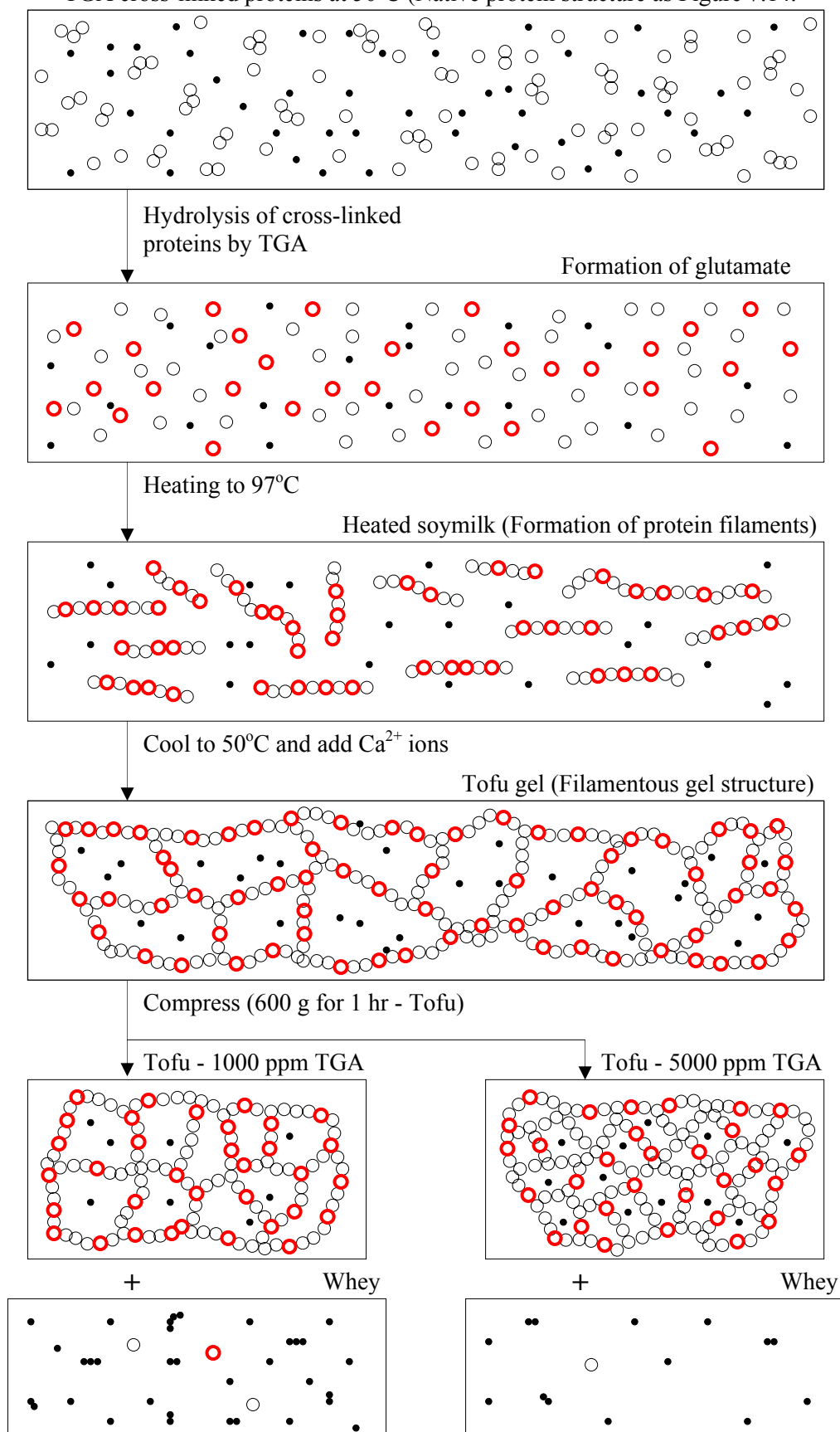


Figure 7.6: A general gelation mechanism for tofu treated with TGA before soymilk boiling. (o) and (●) are representative of high and low molecular weight proteins, respectively. (○) is a glutamate. Adapted from Kao et al. (2003).

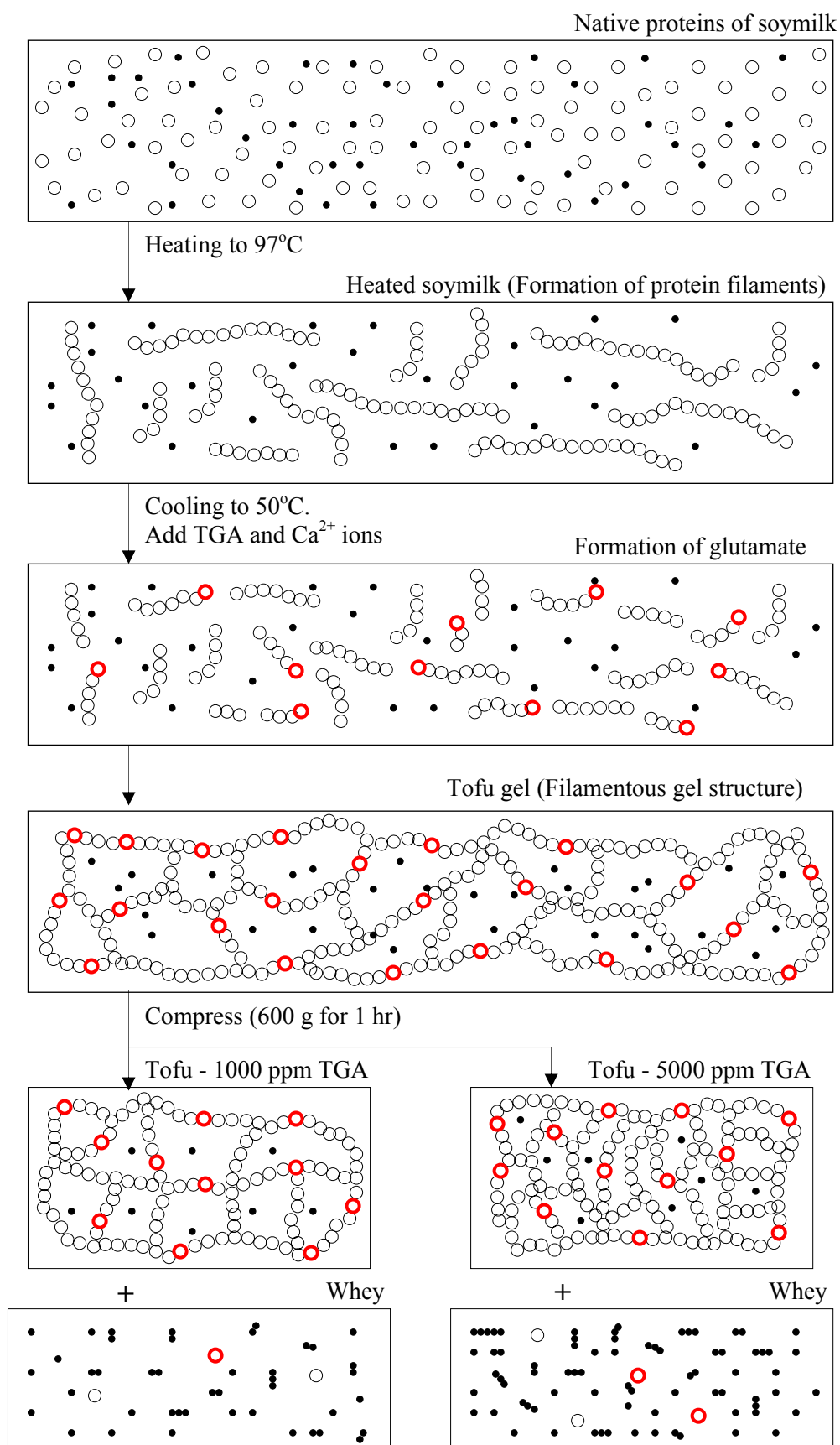


Figure 7.7: A general mechanism of tofu treated with TGA after soymilk boiling. (o) and (●) are representative of high and low molecular weight proteins. (○) is a glutamate. Adapted from Kao et al. (2003).

7.6 Conclusion

The microstructure of the tofu network was found to correlate with changes in the fracture force. A microstructure consisted of a finer-stranded network with less porosity, as observed in glutaraldehyde control tofu, correlated with a higher fracture force. On the other hand, a network with a higher porosity, as observed in TGA control tofu, correlated with a lower fracture force. Although they were different in fracture force, the SDS-PAGE showed that they had similar electrophoretic profiles suggesting that individual soy proteins did not change in the control tofus, but their aggregated form had a very different microstructure.

The microstructures of tofu formed after treatment with low and high concentrations of glutaraldehyde were compared. As a general observation, low concentrations consisted of a more compact structure with smaller pore sizes, whether glutaraldehyde was added before or after soymilk boiling. Compared to the control, these structures resulted in a higher fracture force with treatment before boiling, and a slightly lower fracture force with treatment after boiling. By using high concentrations, the microstructure consisted of a more porous network with larger pore sizes, which significantly lowered the fracture force. Soy proteins were cross-linked with glutaraldehyde to various degrees as shown in SDS-PAGE profiles, suggesting that Maillard-type cross-linking led to a change in properties. However, it is likely that protein glycation also played a significant role.

In the TGA treatment, a significantly higher fracture force was correlated with a fine-stranded network and a compact structure of tofu. The microstructure was different to that of the control, which had a lower fracture force. The microstructure was more similar to the control from the glutaraldehyde treatment. On SDS-PAGE, TGA showed only a small degree of cross-linking. The hydrolysis of the ϵ -(γ -glutamyl)lysyl bonds or glutamine residues was hypothesised as the main reaction occurred during TGA catalysis, with treatment either before or after soymilk boiling, changing the isoelectric point of the soy proteins and leading to a change in aggregation properties.

These results suggest cross-linking is not the major influence on tofu texture, but that modification of soy proteins during processing may lead to a range of microstructures and fracture forces.

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CHAPTER 8

SUMMARY AND CONCLUSION

Treatment with glutaraldehyde and TGA had a profound effect on the textural properties of tofu. Since tofu is formed by heat-induced gelation, it was hypothesised that introduction of cross-linking reagents at various times during processing would provide a very useful tool in manipulating tofu texture.

In the initial investigation, soy proteins were characterised in order to understand the physico-chemical properties of fractionated and unfractionated proteins, and any difference in behaviour with respect to reaction with glutaraldehyde and TGA. Glutaraldehyde, formaldehyde and glyceraldehyde cross-linked unfractionated and fractionated soy proteins to various degrees. The concentration and temperature played a role in the degree of reactivity. Glutaraldehyde was the most reactive molecule, followed by formaldehyde and glyceraldehyde.

TGA also cross-linked unfractionated and fractionated soy proteins. At room temperature, the reactivity of cross-linking depended on the TGA concentration. As expected, higher concentrations resulted in higher reactivity. These results confirmed that soy proteins are substrates for TGA catalysis, and that β -conglycinin was more susceptible to cross-linking than glycinin.

The textural properties of a tofu gel were tested empirically using mechanical compression, mimicking a chewing movement. Prior to testing, a standard procedure of tofu manufacturing in laboratory was developed in order to minimise a number of variables. Three major factors were examined: i) a process from soybean to tofu, ii) a tofu mould, and iii) setting-up of the Instron machine. By controlling all parameters throughout the procedure and assessing the mass balance, all variables were minimised and a consistent product was manufactured. It was expected that any changes observed during testing of the texture of treated-tofu would be attributable to protein cross-linking.

When glutaraldehyde and TGA were added before soymilk boiling or after soymilk boiling, prior to tofu manufacturing, the texture of tofu changed. Glutaraldehyde cross-linked soy proteins as judged by extraction of the proteins from the tofu and analysis by SDS-PAGE. However, increasing the extent of the cross-linking reaction by increasing concentration did not improve the texture as expected. Only a small number of cross-linked proteins improved the texture of tofu, in particular if added before soymilk boiling. High concentrations gave a less firm texture. There was no obvious correlation between cross-linking and fracture force, suggesting that other glycation chemistry may have also played a factor and altered the aggregation properties of the proteins.

Upon TGA treatment, very few cross-links were introduced and yet the texture changed significantly. Furthermore, introduction of cross-links into tofu by TGA increased the fracture force, whereas introduction of cross-links by glutaraldehyde decreased the fracture force. This suggests that other factors had a greater influence on the tofu texture than cross-linking per se. Deamidation of glutamine to glutamic acid by TGA is postulated. By increasing the number of negative charges in the proteins, the solubility is improved and subsequently the functional properties of food proteins are changed (Hamada 1992). A small quantity of deamidation such as 2-5% could result in a significant improvement of protein functional properties (Matsudomi 1985).

The fracture force did not correlate with moisture content. Changes in network structure are thought to contribute to the texture and were examined by SEM. A general observation using SEM on the network structure indicated that a network with a fine-stranded network, compact structure and less porosity give a more firm tofu with higher fracture force (Kao et al. 2003, Nakamura et al. 1984). A relatively larger pore suggests less efficient in protein aggregation.

Although this study demonstrated proof of principle that significant changes in the texture of tofu could be achieved, it must be emphasised that the specific chemical employed would not necessarily be appropriate as food ingredients. In this regard, US Food and Drug Administration (FDA) listed glutaraldehyde as an ingredient that can be directly added to food with certain restriction, although it is not permitted in other countries. For TGA, the enzyme is safe to be added to a food system.

The findings from this study raise a number of questions that need to be explored further. One of the important questions would be; what is the actual reaction occurred that changed the texture of tofu in a real food system. What is the actual role of protein, or fat, or other chemical components in soy food system? The microstructure should be looked beyond SEM technique, such transmission electron microscope (TEM) or confocal laser scanning microscopy (CLSM), to observe which of the changes in the structure are artifacts of SEM preparation and which are genuine reflection of changes in structure of the hydrated tofu.

In conclusion, the findings upheld hypotheses i) and ii), but not to hypotheses iii). This study shows that the textural properties of tofu can be altered through glutaraldehyde and TGA treatment. Surprisingly, the cross-linking by glutaraldehyde did not result in producing a more firm tofu, in contrast to the cross-linking occurred in wheat proteins (Gerrard et al. 2003). Nevertheless, both treatments offer a potential method for food manufacturers to produce soy products that are tailored to consumer preference.

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CHAPTER 9

EXPERIMENTAL

9.1 General methods

9.1.1 Materials and apparatus

Unless otherwise stated, all chemicals, reagents and solvents were obtained from Sigma-Aldrich New Zealand Ltd. (Auckland, New Zealand) or BDH Chemicals New Zealand, Ltd. (Palmerston North, New Zealand) and were generally analytical grade.

Transglutaminase (TGA) was obtained from Amcor Trading Pty. Ltd. (Sydney Australia). The commercial preparation consisted of 20% protein and 80% dextrin filler.

Defatted soy flour (52% protein content), bovine serum albumin and bromophenol blue were purchased from Sigma Chemical Company Ltd. (MO, USA).

Soybeans were purchased in a single batch from the Asian Food Warehouse, Christchurch, New Zealand and were of Chinese origin.

The dialysis tubing had a 12-14000 Dalton cut-off and was purchased from Medical International Ltd (London, England).

Anti-foaming agent (BDH 1510 Silicone Antifoam) was purchased from BDH Laboratory Supplies (Poole, England).

A standard molecular weight protein marker was purchased from Sigma Chemical Company Ltd. (MO, USA), referred as Sigma marker as shown in Table 9.1.

Top pan balance used Sartorius (Goettingen, Germany).

Table 9.1: Standard molecular weights of the proteins in Sigma marker.

Protein	Molecular weight (kDa)
Rabbit muscle myosin	205
<i>E. coli</i> β -galactosidase	116
Rabbit muscle phosphorylase b	97
Rabbit muscle fructose-6-phosphate kinase	84
Bovine serum albumin	66
Bovine liver glutamic dehydrogenase	55
Ovalbumin	45
Rabbit muscle glyceraldehyde-3-phosphate	36
Bovine erythrocyte anhydrase	29
Bovine pancreas trypsinogen	24
Soybean trypsin inhibitor	20
Bovine milk α -lactalbumin	14
Bovine lung aprotinin	6.5

9.1.2 General laboratory practice

All pH measurements were performed using an EDT Instrument BA 350 series 3 pH meter fitted with an EDT Instrument E8030 electrode, calibrated against standard buffers at pH 4.0, pH 7.0 and pH 9.0.

High-speed centrifugation was performed in an Eppendorf Centrifuge (Model 5810R). A rotor (Eppendorf Model F34-6-38, 6 x 125 g, max 12000 rpm) was used for centrifugation of sample more than 50 mL. Low speed centrifugation was achieved using a bench-top microcentrifuge supplied by Qualitron Inc. (OH, Holland).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was routinely performed using a Bio-Rad 300 Power Pack. Gradipore pre-cast acrylamide gels, 4-20% gradient (Gradipore Ltd., Frenchs Forest, NSW, Australia) were run using a Bio-Rad i-gel kit.

UV absorbance measurements were taken with a Bio-Rad SmartSpec 3000 spectrophotometer.

For both RP-HPLC and SE-HPLC, a Waters W2690 solvent delivery/control/sample injector system and a Waters 2487 UV-visible detector were used. High performance liquid chromatography (HPLC) analyses were carried out using Waters HPLC system. Water for HPLC was produced with a Milli-Q Water Purification System (Millipore).

Fats in dried soymilk and tofu were extracted using n-hexane in a standard laboratory Soxhlet apparatus using extraction thimble 30 mm x 100 mm (Whatman, Maidstone, England).

Densitometric analysis of SDS-PAGE analyses used Phoretix 1D Plus Software owned by Crop & Food Research, Lincoln, New Zealand.

All cross-linking reactions of proteins *in vitro* were carried out in Eppendorf tubes. A ventilated oven was used for temperature-controlled incubations.

A Leica S440 scanning electron microscope (Wetzlar, Germany) was used for microstructure examination.

An Instron Universal Testing Machine (Model 444, Canton, MA, USA) fitted with a GPIB interface was used for computer-controlled operation. An in-house program written in LabVIEW Version 7.11 (National Instrument, Austin, TX, USA) was developed to control the probe speed (Crop & Food Research, Lincoln, New Zealand). Data generated from the Instron UTM were analysed using the macro program written in an MS Excel Visual Basic for Application (Crop & Food Research, Lincoln, New Zealand) to determine the maximum distance, fracture force and compression modulus as demonstrated in Figure 5.2 (Chapter 5).

9.1.3 Buffers

0.5% SDS-0.05 M phosphate buffer, pH 6.9: 3.53 g of Na_2HPO_4 and 3.39 g KH_2PO_4 were mixed together in 500 mL of distilled water to the pH 6.9. Then, 5.0 g SDS was added, and then the mixture was stirred and gently heated in a microwave oven until completely dissolved.

5 x Tank buffer: 45 g Tris, 216 g glycine and 15 g SDS were dissolved in 3 L distilled water and stored at 4°C. The solution was diluted 1 in 5 with distilled water before use.

2 x Treatment buffer – reduced: 125 μL of 1 M Tris-HCl pH 6.8, 2 mL 10% SDS (w/v), 1 mL glycerol, 500 μL 2-mercaptoethanol and 125 μL 1% bromophenol blue (w/v) were dissolved in 750 μL distilled water and stored at -10°C. The solution was diluted 1 in 2 with distilled water before use.

2 x Treatment buffer – non-reduced: As for 2 x Treatment buffer (reduced), but without 2-mercaptoethanol. This was replaced with distilled water.

0.1% Coomassie brilliant blue stain: 0.1 g Coomassie brilliant blue, 10 mL glacial acetic acid and 50 mL methanol were dissolved in 40 mL distilled water. The solution was stirred for approximately 20-30 min, and subsequently filtered and stored at room temperature (20°C).

Destaining solution (5% methanol and 10% acetic acid): 100 mL acetic acid glacial and 50 mL methanol were diluted in 850 mL distilled water. The solution was stored at room temperature (20°C).

9.1.4 Tofu manufacture

Wet soybeans were blended using a kitchen blender (Kambrook, Power Plus Blender).

The soymilk was extracted from the slurry using a juice extractor (Braun) and filtered through muslin cloth.

A coagulant was prepared by dissolving 6.7 g $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ (Sigma) in 50 mL of water.

9.1.5 A standard condition for running SDS-PAGE gel

Protein samples (20 μL) were added to treatment buffer (reducing or non-reducing, 20 μL) in Eppendorf tubes. The mixtures were vortexed and then centrifuged briefly in order to return the liquid to the bottom of the tubes. Tubes were then incubated boiling water for 2 min, and subsequently, a 20 μL of mixture was loaded onto the 4-20% gradient pre-cast polyacrylamide gel (Gradipore). The electrophoresis was run at a constant volt (150 V) for 1.5 hour or until the blue line of bromophenol blue reached the green line of the Bio-Rad i-gel kit. After completion, the gel was stained with 0.1% Coomassie brilliant blue for 30 min, and destained with destaining solution overnight. The gel was then digitally photographed using a Nikon D1X camera fitted with a Nikon 60 mm macro lens for densitometric analysis. All SDS-PAGE analyses were carried in duplicate.

9.1.6 Determination of protein concentration

The protein concentrations were assessed using the Bradford method (Bradford 1976). A standard curve plotting absorbance versus concentration was obtained using bovine serum albumin in the concentration range 2 $\mu\text{g/mL}$ to 12 $\mu\text{g/mL}$ as presented in Figure 9.1.

Protein samples were dissolved in water and diluted if necessary in order to fall in the linear range of the standard curve. 100 μL of each sample was added to cuvettes containing 700 μL of distilled water, then 200 μL of Bio-Rad Bradford reagent were added and then the content of the cuvette mixed manually. The mixture was left at room temperature (20°C) for 6 min and subsequently the absorbance was read at 595 nm

against distilled water blank. Subsequently, the concentration of proteins was quantified by comparison to the standard curve.

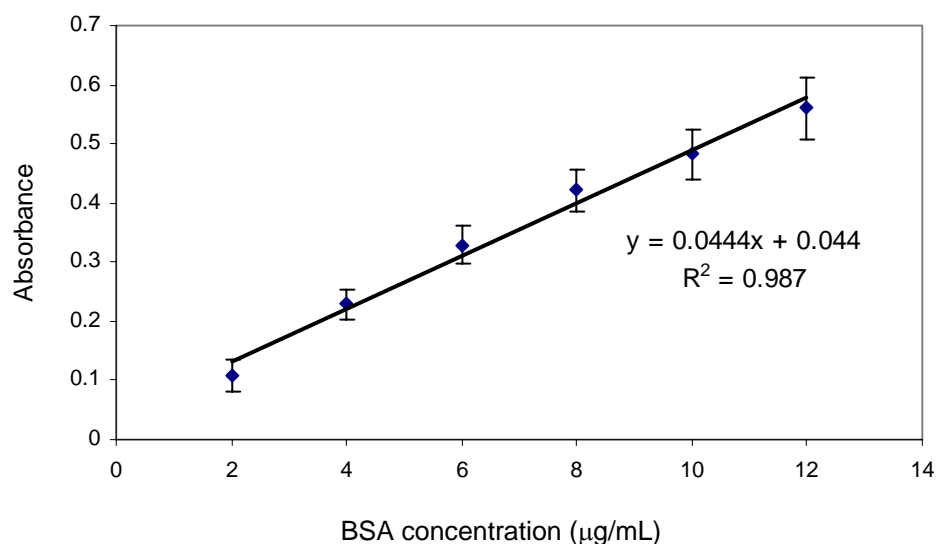


Figure 9.1: A standard curve of absorbance versus bovine serum albumin (BSA) concentration. Values are the means of triplicate measurements. Error bars represent the standard error of the means.

9.1.7 Statistical analysis

The significance of differences between means was determined by paired sample t-test using the SPSS statistical package (SPSS 2003). The levels of significance used were 95% and 99%.

9.2 Characterisation of soy proteins (Chapter 2)

9.2.1 Comparing the extraction methods

Four methods were tested to isolate glycinin and β -conglycinin fractions from defatted soy flour and subsequently subjected to SDS-PAGE. These methods were adapted from Ji et al. (1999), Scilingo and Anon (1996), Thanh et al. (1975) and

Peterson and Wolf (1988). These methods were schematically outlined in section 2.2, chapter 2. These methods were carried out in duplicate.

In this initial testing, the glycinin and β -conglycinin fractions were not quantified but a qualitative assessment was made by comparing SDS-PAGE analysis of each method. The Peterson and Wolf (1988) method was selected, and all resulting extracts from this method were quantified in order to evaluate the reliability and efficiency of the chosen method.

i) Scilingo and Anon (1996) method: 10 g of defatted soy flour were dissolved in 100 mL distilled water. The pH of the solution was adjusted to 8.0 with 2 M NaOH and the solution stirred at room temperature for 2 hr. Then, the mixture was filtered through a gauze (~ 8 mesh) and centrifuged at 11000 rpm for 30 min at 4°C. After centrifugation, the supernatant was collected. Subsequently, the pH of the collected supernatant was adjusted to 6.4 with 2 M HCl to precipitate the glycinin fraction, and was left overnight at 4°C. Then the mixture was centrifuged at 11000 rpm for 30 min at 4°C to separate the precipitate and supernatant.

The precipitate was then washed twice with 10 mL of distilled water. After each washing, the solution was centrifuged at 11000 rpm for 30 min at 15°C. The washed precipitate was then resuspended at pH 8.0 with 2 M NaOH. This solution was freeze-dried to yield the glycinin fraction.

For the resulting supernatant, the pH was adjusted to 4.8 with 2 M HCl to precipitate the β -conglycinin fraction. The solution was stirred for 2 hr at room temperature. After stirring, this solution was centrifuged at 3300 rpm for 10 min at 15°C. The precipitate was collected and washed twice with 10 mL distilled water. After each washing, the solution was centrifuged at 11000 rpm for 30 min at 15°C. The washed precipitate was then resuspended at pH 8.0 with 2 M NaOH. This solution was freeze-dried to yield the β -conglycinin fraction.

ii) Peterson and Wolf (1988) method: Two types of extraction buffer were prepared as follows.

Buffer A: 0.03 M Tris-HCl, 10 mM 2-mercaptoethanol, pH 8.0.

Buffer B: 0.4 M NaCl, 33 mM K_2HPO_4 , 2.6 mM KH_2PO_4 , 0.02% NaN_3 , 10 mM 2-mercaptoethanol, pH 7.6.

10 g of defatted soy flour were dissolved in 100 mL of Buffer A. The solution was stirred for 1 hr at 20°C. After stirring, the solution was transferred into a centrifuge tube and centrifuged at 11000 rpm for 20 min at 4°C. The resulting precipitate was freeze-dried (Precipitate P1). The remaining supernatant was adjusted to pH 6.4 with 2 M HCl and cooled overnight at 4°C. The cooled supernatant was centrifuged at 13300 rpm for 20 min at 4°C. The resulting precipitate and supernatant were separated, containing crude glycinin and β -conglycinin respectively.

The precipitate, consisting of crude glycinin, was washed twice with 10 mL of 30 mM Tris-HCl, pH 6.4. The mixture was centrifuged at 11000 rpm for 10 min at 4°C after each washing. After centrifugation, the supernatant was freeze-dried (Supernatant T1), then dissolved with 10 mL of Buffer B dialysed against distilled water for 3 hrs at 4°C. Finally, the dialysed solution was freeze-dried to obtain the glycinin fraction.

The pH of supernatant, containing the crude β -conglycinin was adjusted to 4.8 with 2 M HCl. Then the solution was stirred for 1 hr at 20°C. Subsequently, this solution was centrifuged at 11000 rpm for 10 min at 15°C. The resulting supernatant was dialysed against distilled water for 3 hrs at 4°C, then freeze-dried (Supernatant S1).

The resulting precipitate (from Supernatant S1) was dispersed in 40 mL 30 mM Tris-HCl at pH 7.6. Then the pH was adjusted to 6.2 with 2 M HCl. Subsequently, the solution was centrifuged at 11000 rpm for 10 min at 15°C. The resulting supernatant was then dialysed against distilled water for 3 hrs at 4°C and freeze-dried to obtain the β -conglycinin fraction.

The remaining precipitate (from β -conglycinin fraction above) was washed with 40 mL 30 mM Tris-HCl, pH 7.6 and then the pH was adjusted to 6.2 with 2 M HCl. The solution was dialysed against distilled water for 3 hr at 4°C, and centrifuged at 11000 rpm for 10 min at 15°C. The resulting supernatant (Supernatant S2) and the precipitate (Precipitate P2) were freeze-dried.

Since this method was chosen as a method for extracting glycinin and β -conglycinin fractions for other experiments, this method was repeated three times and all the extracts obtained from defatted soy flour; namely P1, glycinin, T1, β -conglycinin, S1, S2 and P2 were quantified. The protein concentration in each fraction was assessed. The results are presented in Table 2.1 Chapter 2.

iii) Thanh et al. (1975) method: Three types of extraction buffers were prepared and used in this method.

Buffer 1: 63 mM Tris-HCl containing 10 mM 2-mercaptoethanol, pH 7.8.

Buffer 2: 63 mM Tris-HCl containing 10 mM 2-mercaptoethanol, pH 6.6 at 2-3°C.

Buffer 3: 0.4 M NaCl, 33 mM K_2HPO_4 , 2.6 mM KH_2PO_4 , 0.02% NaN_3 , 10 mM 2-mercaptoethanol, pH 7.6.

10 g of defatted soy flour were dissolved in 150 mL Buffer 1. The mixture was stirred for 1 hr at room temperature, and subsequently centrifuged at 10000 rpm for 15 min at room temperature. The precipitate was discarded and the supernatant was collected.

The pH of the resulting supernatant was adjusted to 6.6 with 2 M HCl to precipitate the glycinin. The mixture of supernatant and precipitate was dialysed against Buffer 2 at 4°C for 3 hr. After dialysis, the mixture was centrifuged at 10000 rpm for 20 min at room temperature to separate the precipitate and supernatant.

The precipitate was then dissolved in Buffer 3 and freeze-dried to yield the glycinin fraction. Meanwhile, the pH of the supernatant was adjusted to 4.8 with 2 M HCl to precipitate the β -conglycinin. The mixture was centrifuged at 10000 rpm for 20 min at room temperature. The precipitate was collected and subsequently dispersed in

distilled water. The pH of the solution was adjusted to 7.0 with 2 M NaOH while stirring. After pH adjustment, the solution was freeze-dried to yield the β -conglycinin fraction.

iv) Ji et al. (1999) method: 10 g of defatted soy flour were dissolved in 100 mL distilled water. The solution was stirred for 2 hr at room temperature (20°C). After stirring, the solution was cooled to 4°C for 3 hr. It was then centrifuged at 10000 rpm for 25 min at 4°C. The resulting supernatant was collected and the precipitate, which contained the glycinin fraction, was freeze-dried.

The supernatant was collected and the pH was adjusted to 4.8 with 1 M HCl to precipitate the β -conglycinin fraction. The mixture was then centrifuged at 3300 rpm for 10 min at 15°C. The precipitate was then collected and freeze-dried to produce the β -conglycinin fraction.

9.2.2 SDS-PAGE analysis

SDS-PAGE was carried out on defatted soy flour and all fractions obtained from the Peterson method (Peterson and Wolf 1988). Three mg of defatted soy flour and 1 mg of each fraction of glycinin, β -conglycinin, P1, T1, S1, S2 and P2 were dissolved in 500 μ L of distilled water. The solutions were vortexed and centrifuged at 14000 rpm for 5 min at 20°C. SDS-PAGE analysis was carried out as described in Figure 9.1.5. All SDS-PAGE analyses were carried out in triplicate.

9.2.3 Densitometric analysis

The gel image was analysed by Phoretix 1D Plus Software (Crop & Food Research, Lincoln, New Zealand) for quantification of protein subunits. The areas of protein subunits were marked and the interference backgrounds were subtracted from the image. The areas of the subunits were measured and related to the relative percentage of each band in the protein sample. All measurements were carried out in triplicate.

9.2.4 RP-HPLC analysis

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) of defatted soy flour, glycinin and β -conglycinin was adapted from the method of Peterson and Wolf (1992). All chromatograms were run in triplicate. Prior to injection, the protein samples (defatted soy flour, glycinin and β -conglycinin) were prepared in the same way as those in the procedures described in section 9.2.2.

The column used was a 150 x 4.6 mm Zorbax 300SB-C8 fitted with a 12.5 x 4 mm Zorbax 300SB-C8 guard column. Solvents used were (A) water containing 0.1% (v/v) trifluoroacetic acid (TFA) and (B) acetonitrile containing 0.1% (v/v) TFA. Deaeration was achieved by vacuum filtration through a 0.22 μ m filter, rapid sparging with helium (100 mL/min for 10 min) and constant slow bubbling of helium into capped, vented solvent reservoirs (30 mL/min). Samples of 10 μ L were injected on to the column, which was maintained at 50°C, and proteins were eluted using a two-step solvent gradient (20-33% B over 13 min, then 33-45%B over 48 min) at a flow rate of 1.0 mL/min. The column was returned to the initial solvent composition over 1 min and re-equilibrated for 10 min prior to the next analysis. Eluted components were detected by peptide bond absorption at 210 nm. Instruments were controlled and the chromatographic traces were recorded using a personal computer running the Waters "Empower" software package.

9.2.5 SE-HPLC analysis

Only glycinin and β -conglycinin fractions were characterised by SE-HPLC. One mg of glycinin and β -conglycinin was dissolved in 500 μ L of distilled water prior to injection onto the column.

SE-HPLC conditions were adopted from a method of Sutton et al. (2003). The column used was a BioSep SEC-4000S (300 x 7.5 mm) with a BioSep SEC-4000S guard column (75 x 7.5 mm). Solvents used were (A) water containing 0.1% (v/v) trifluoroacetic acid (TFA) and (B) acetonitrile containing 0.1% (v/v) TFA. Deaeration was achieved by vacuum filtration through a 0.22 μ m filter, rapid sparging with helium

(100 mL/min for 10 min) and constant slow bubbling of helium into capped, vented solvent reservoirs (30 mL/min). Samples (10 μ L) were injected onto the column, which was maintained at 20°C and proteins were eluted isocratically at a solvent composition of 50% A:50% B for 35 min at a flow rate of 0.5 mL/min. Eluted components were detected by peptide bond absorption detected at 210 nm and the chromatographic traces were recorded on a personal computer using the Waters "Empower" software package.

9.3 The cross-linking of soy proteins (Chapter 3 and Chapter 4)

9.3.1 Monitoring cross-linking by SDS-PAGE and densitometry

Defatted soy flour, glycinin and β -conglycinin were employed for each cross-linking experiment. For chemical cross-linking, they were incubated with glutaraldehyde, formaldehyde, glyceraldehyde, whilst enzymatic cross-linking employed transglutaminase (TGA). All incubations were carried out in an Eppendorf tube. The experimental designs are presented in Chapters 3 and 4.

At all times, *in vitro* incubations used distilled water at pH 6.9, to corroborate with the preparation tofu, which used distilled water. In the preliminary investigation, the SDS buffer, pH 6.9 was also used. However, the SDS-PAGE profiles and protein solubility were similar to those of distilled water. A total of 40% protein of defatted soy flour was dissolved in both solvents. Therefore, distilled water was chosen for the following experiments.

Chemical cross-linking: Defatted soy flour (6 mg) was dissolved in 600 μ L distilled water and vortexed. The solution was then centrifuged at 11000 rpm for 5 min at room temperature. A series of concentrations of the reagents was prepared namely: 1 mM and 2 mM glutaraldehyde; 50 mM and 100 mM formaldehyde; and 50 mM and 100 mM glyceraldehyde. The final volume of incubation was made up to 1 mL with distilled water. The glutaraldehyde experiment was carried out at a range of temperatures, 20, 30, 40 and 50°C, whilst the formaldehyde and glyceraldehyde experiment was carried out at 30 and 50°C.

For glycinin and β -conglycinin, 2 mg of each fraction was dissolved in 600 μ L of distilled water in Eppendorf tubes, and reacted using the same reagents concentrations and incubation temperatures used as described for defatted soy flour.

TGA cross-linking: Defatted soy flour solution (6 mg in 600 μ L distilled water), glycinin and β -conglycinin (both at 2 mg in 600 μ L distilled water) were prepared in Eppendorf tubes. To this, an appropriate volume of TGA stock solution (5000 ppm) was added for 500 ppm and 1000 ppm TGA concentration. The final volume was adjusted to 1 mL. Incubation was carried out at 20°C.

For chemical and TGA cross-linking, samples were removed at sampling intervals as indicated in Table 3.2 (Chapter 3) and Table 4.1 (Chapter 4). A 20 μ L aliquot was pipetted out at each interval and immediately cooled in ice water. The electrophoresis was routinely run as described in Section 9.1.5. All treatments were carried out in duplicate gels of duplicate treatments.

The gels were digitally photographed and images were scanned with Phoretix 1D Plus software for densitometric analysis. The area of each subunit was marked and the area under the densitogram was measured. The total area under the densitogram represented the total amount of protein per 20 μ L loading volume. The peak areas represent the composition of protein subunits loaded on the gel. Thus, the concentration of each protein subunit at every interval was quantified to determine the relationship between protein loss and incubation time. The concentration of remaining protein was converted to \ln concentration, and then \ln concentration was plotted versus incubation time to fit a linear equation of first-order. The reaction rate was estimated by the slope of the linear equation. This procedure was applied to both chemical and enzymatic cross-linking.

9.3.2 Monitoring cross-linking by SE-HPLC

Cross-linked defatted soy flour was not examined by SE-HPLC because it precipitated and interfered with the column. TGA cross-linked samples formed precipitates very rapidly and were also unsuitable for SE-HPLC analysis. Only the

glycinin and β -conglycinin incubations were analysed by SE-HPLC. Both were incubated with 1 mM glutaraldehyde at 20°C. The sample preparation was described in section 9.3.1. After injection, the chromatogram profile of cross-linked proteins was monitored starting from 0 min incubation. The injection interval was automatically programmed at 40 min. A total of 20 injections were performed.

The SE-HPLC running conditions were described as in section 9.2.5. The SE-HPLC column was calibrated using protein standards with a range of molecular sizes as follows: bovine serum albumin (66 kDa), β -amylase (200 kDa) and thyroglobulin (670 kDa). The quantification of protein subunits over the course of the incubation was based on the peak area. All experiments were carried in duplicate.

9.4 Method development for tofu manufacture (Chapter 5)

9.4.1 Setting up standard condition for Instron UTM

An Instron UTM was used to deform the sample by a uniaxial compression (Hou and Chang 1998, Wang and Chang 1995). Prior to measurements on test samples, the instrument set-up, methodology and sample preparation were standardised.

In order to fix the variables, a number of tests were carried out as described in section 5.2.1 (Chapter 5). In preliminary experiments, the surface for tofu compression was lubricated with glycerine, the plunger speed was fixed at 100 mm/min and the sample height was trailed at 26 mm (full height) and 13 mm (half height) using commercial Chines tofu. All experiments were carried out in four replications.

In measuring the texture, the fracture force and compression modulus were measured. The force-deformation curve generated from the Instron UTM (Bourne 1978) was analysed using an MS Excel Visual Basic for Application (Crop & Food Research, Lincoln, New Zealand) macro to determine the fracture distance, fracture force and compression modulus as shown in Figure 5.2 (Chapter 5).

9.4.2 Standard procedure for tofu manufacture – Control tofu

The standard procedure of tofu manufacture was based on the method of Cai et al. (1997). The preparation for control tofu is described as follows.

Dried soybeans (150 g) were soaked with water for 10 hr at room temperature. After soaking, the wet beans were rinsed. The wet beans were weighed (typically 326 g) and subsequently blended with 730 mL of warm water (25-30°C) using a kitchen blender (Kambrook) at high speed for 2 min. After blending, the slurry was juiced out with a juice extractor (Braun) and then filtered through a muslin cloth to obtain the soymilk. The volume of soymilk was measured and found in the range of 740-750 mL.

To the soymilk, 20-30 mL of water was added to make the volume up to 770 mL and one drop of anti-foaming agent was also added. Subsequently, this soymilk was heated moderately with constant stirring in the beaker to 97°C on a Chiltern magnetic stirrer hotplate (Chiltern, Auckland, New Zealand) with heating set at 7. During heating, the beaker was covered with aluminium foil to minimise water evaporation. When the temperature of the soymilk reached 97°C, the temperature was held for 5 min before the beaker was removed from the hot plate, and allowed to cool to 87°C.

Upon reaching 87°C, 750 mL hot soymilk were measured (or if the sample was less than 750 mL, hot water was added to make the volume up to 750 mL) and poured in a beaker simultaneously with 50 mL coagulant (containing 6.7 g $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ in 50 mL water). The coagulation was left for 8 min to allow the soymilk to form a gel. This gel was then transferred into the mould and compressed with a 600 g (or 900 g) load for 1 hr. After removing the load, the tofu was allowed to remain standing in the mould and left overnight at room temperature (20°C), prior to further testing (replication stated under each Figure or Table).

9.4.3 Tofu mould

The prototype of tofu mould design was adapted from (Byun et al. 1995). Due to inconsistencies in the resulting tofu, the mould design was improved to the third design, as discussed in section 5.3 (Chapter 5).

9.4.4 Mass balance assessment

The volume, weight, water and protein content in soymilk, tofu and whey were assessed. The volume and weight were measured using measuring cylinder and top pan balance.

Moisture content: The moisture content of the sample was determined by a gravimetric method (AOAC 1984). 5 g sample was weighed and dried in the ventilated laboratory oven at 105–110°C for 24 hours. The moisture content was calculated using the following equation.

$$M_n = (W_w - W_d)/W_w \times 100\% ;$$

where M_n = moisture content (%) of material, W_w = wet weight of the sample and W_d = weight of the sample after drying.

Protein content: Protein contents of samples were determined by the Dumas Method using a LECO CNS-2000 Analyser (Laboratory Equipment Corporation Ltd, MI, U.S.A.) calibrated with EDTA for % nitrogen. A freeze-dried sample (0.31 g) was loaded into a ceramic boat sample holder. The samples were combusted at 1050°C in a stream of pure oxygen and the nitrogen gas from the reduced nitrogen oxide gases was analysed by a thermal conductivity analyser. Since the nitrogen was determined by the Dumas method and the instrument output was in % nitrogen, a universal conversion factor of 6.25 x % nitrogen was adapted for the calculation of protein content on a dry weight basis (AOAC 1996).

Density: The density of tofu was determined by measuring water displacement using a measuring cylinder. A tofu sample (10 g) was placed in the water (~ 25°C) and the water displacement (volume) was read. The value was calculated using the formula below

$$\rho = m/v$$

where m = mass and v = volume of water displacement.

9.5 Measuring textural properties of treated-tofu (Chapter 6)

9.5.1 Preparation of glutaraldehyde-treated tofu

Glutaraldehyde stock solution: The concentration and the volume of glutaraldehyde added to the soymilk were prepared according to Table 9.2.

Soymilk: The preparation of soymilk was in the same way as described in section 9.4.2.

Control tofu: The preparation of control tofu was in the same way as described in section 9.4.2. Instead of adding 30 mL glutaraldehyde stock solution, 30 mL of distilled water was added at room temperature.

Table 9.2: The concentration and the volume of glutaraldehyde solutions used. The total volume of soymilk before and after boiling was 740 and 720 mL, respectively.

Treatment (Before and after soymilk boiling)	Volume of glutaraldehyde solution added to soymilk (mL)	Glutaraldehyde stock solution (mM)
1 mM glutaraldehyde	30	27
2 mM glutaraldehyde	30	54
15 mM glutaraldehyde	30	400
30 mM glutaraldehyde	30	800

Glutaraldehyde-treated tofu: The preparation of glutaraldehyde-treated tofu, with the glutaraldehyde added before and after soymilk boiling, is schematically outlined in Figure 9.1. For the treatment before soymilk boiling, 740 mL of soymilk was measured and added to 30 mL glutaraldehyde solution (Table 9.2) for each treatment. One drop of anti-foaming agent was added and the beaker was covered with aluminium foil. The heating, coagulation and compression proceeded as described in section 9.4.2.

For treatment after soymilk boiling, 740 mL of soymilk was heated to 97°C with constant stirring. Upon reaching 97°C, the temperature was maintained for 5 min and

the soymilk was allowed to cool. Once the soymilk reached 87°C, 720 mL was measured out (if necessary the volume was made up with hot water) and poured simultaneously with 30 mL glutaraldehyde solution and 50 mL coagulant into a beaker. The mixture was allowed to coagulate for 8 min to form a tofu gel.

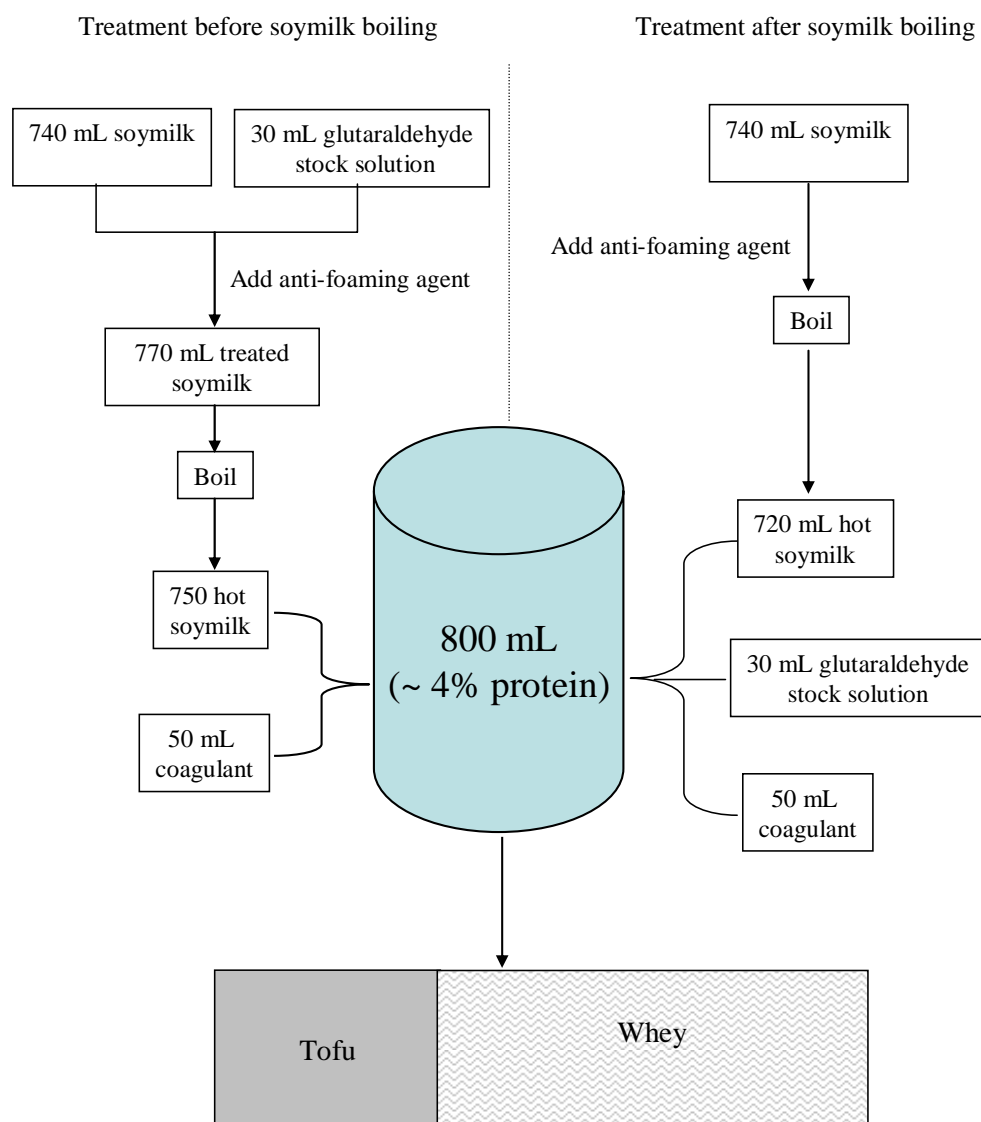


Figure 9.1: Schematic outline of tofu manufacture including glutaraldehyde treatment.

9.5.2 Preparation of TGA-treated tofu

Preparation of soymilk: The method was as for control tofu in section 9.4.2, but the volume of water used for blending was 740 mL. For treatment both before and after soymilk boiling, 770 mL soymilk was measured (if necessary the volume was made up with water). The schematic outline for the preparation of TGA-treated tofu is shown in Figure 9.2.

Preparation of control tofu for TGA: The preparation of control tofu for TGA treatment proceeded as for the control tofu for glutaraldehyde treatment (section 9.4.2) with the following modifications.

- i) 770 mL of soymilk were heated with constant stirring to 50°C, held at this temperature for 8 min before resuming heating to 97°C with constant stirring, and being held at this temperature for 5 min.
- ii) After heating, the hot soymilk was allowed to cool to 50°C. At this temperature, 750 mL soymilk was measured (if necessary the volume was made up with water) and poured simultaneously into a beaker with 50 mL coagulant.

Preparation of TGA-treated tofu: For the TGA added before soymilk boiling, 32 mg TGA (1000 ppm TGA) or 160 mg TGA (5000 ppm TGA) (weight TGA per weight soy proteins) were added to 770 mL of soymilk at 50°C and held for 8 min to cross-link the proteins. Subsequently, the heating, coagulating, compressing and storing were as for the control tofu for TGA.

For the TGA added after soymilk boiling, the same amount of TGA as with treatment before soymilk boiling was added at 50°C, together with 50 mL coagulant and held for 8 min. The remaining procedure was that of the TGA control tofu.

9.5.3 Mass balance assessment

The measurement of volume, weight, water and protein content of soymilk, tofu and whey, and density of tofu was as for glutaraldehyde-treated tofu in section 9.4.4.

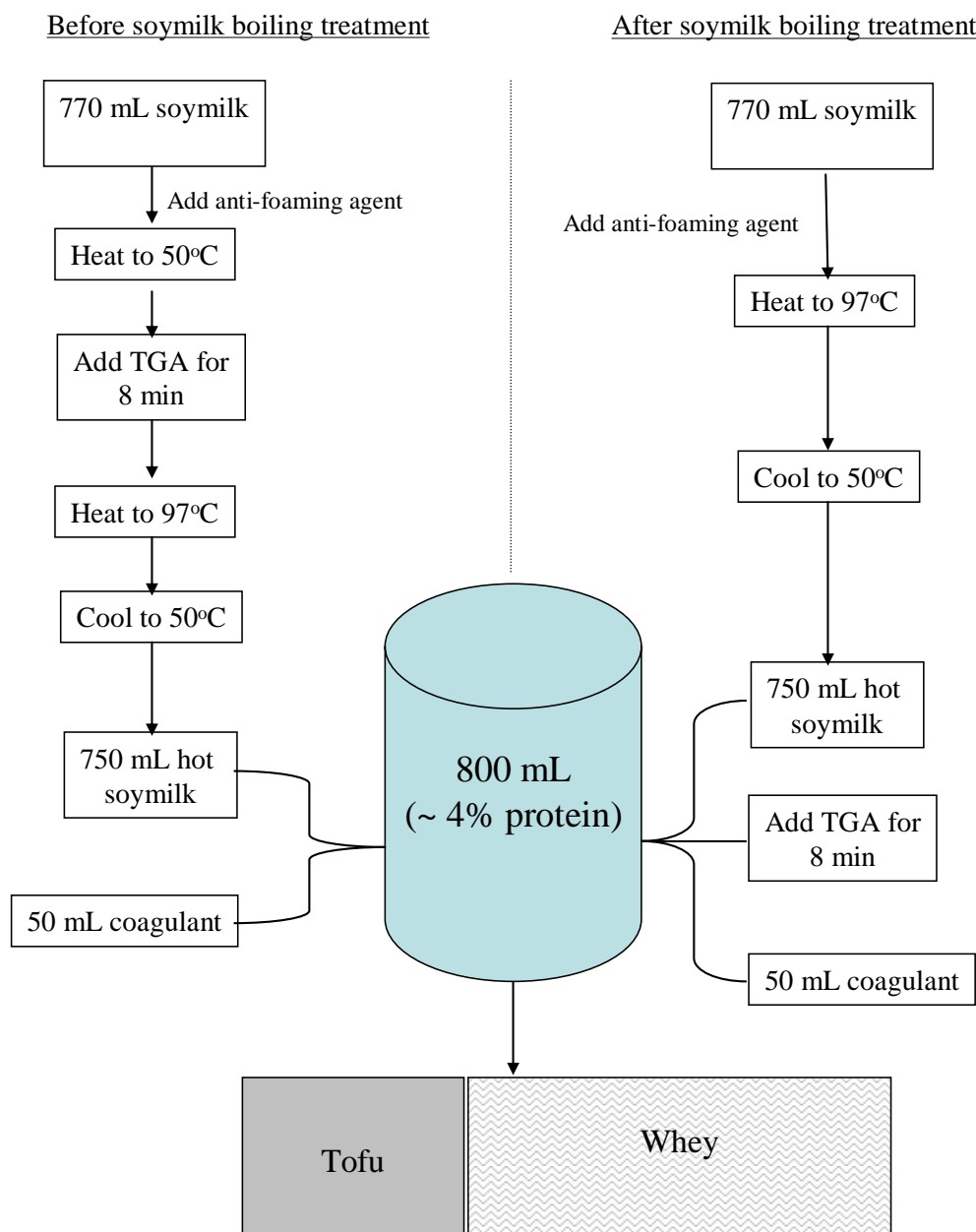


Figure 9.2: Schematic outline of the tofu-manufacture procedure for TGA treatment.

9.5.4 Measurement of textural properties

Instron UTM set up: The Instron UTM was fitted with a crosshead size of 65 mm diameter and attached with a 50 N load cell. The compression distance was programmed at 15 mm (50% deformation). The crosshead speed was fixed at 100

mm/min. At all times, the surfaces of the crosshead and the supporting plate were lubricated with glycerine and the temperature for testing maintained at 20°C.

Tofu sample: The tofu column from each treatment was cut evenly into three portions, top, middle and bottom. The core of the middle portion was cut using a cylindrical cutter (36 mm diameter and 30 mm height). The tofu sample was placed under the crosshead surface for compression.

Texture profile analysis: The Instron UTM automatically generated force-deformation curve. The data point of this curve was converted into an MS Excel program. These data were then analysed using MS Excel Visual Basic Application macro (Crop & Food Research, Lincoln, New Zealand) to measure the fracture distance, fracture force and compression modulus of tofu sample. Typical curves of fracture force and compression moduli versus compression distance are shown in Figure 5.2 (Chapter 5). The maximum distance of compression was determined using this figure. The maximum peak of fracture force and compression modulus curves gives the fracture force and compression modulus of the tofu sample.

9.5.5 SDS-PAGE analysis

Prior to SDS-PAGE analysis, the soymilk, tofu and whey were freeze-dried. Soybean powder, dried soymilk and tofu were subjected to defatting. Approximately 1 g of soybean powder, soymilk or tofu sample was placed in a thimble and then defatted using 100 mL n-hexane in a standard laboratory Soxhlet apparatus, at about 70°C for 4 hrs (Lusas and Riaz 1995). The samples were then dried overnight under aeration in fume cupboard prior to electrophoresis.

Four mg of protein sample (defatted soybean, tofu and soymilk, and whey) was dissolved in a 500 µL urea solution containing 8 M urea, 0.05 M Tris, 0.05 M dithiothrietol (DTT) at pH 10 to extract the proteins (Woods and Orwin 1987). The solution was then vortexed, sonicated for 15 sec and centrifuged at 11000 rpm at room temperature for 5 min. 20 µL of the aliquot was transferred to an Eppendorf tube and prepared for electrophoresis as described in section 9.2.3.

Non-reduced electrophoresis: Non-reduced electrophoresis was carried out on defatted soybean, and soymilk, tofu and whey of control samples of glutaraldehyde treatment. Sample preparation was similar to that for reducing conditions, except no DTT or 2-mercaptoethanol was added at any point of the procedure. No heating was applied prior to loading samples onto the gel. Staining and destaining followed the methods described in Section 9.1.5.

All electrophoresis was carried out in duplicate. The SDS-PAGE gels presented in this thesis are representative of duplicate gels of duplicate extractions.

9.6 Examination of microstructure (Chapter 7)

A Leica S440 electron microscope (Wetzlar, Germany) was used for microscopic scanning of microstructure of glutaraldehyde- and TGA-treated tofu prepared as in section 9.5. A freeze fracture technique was employed to observe the micrograph of internal structure (Goldstein et al. 1992). The method of sample preparation was adapted from a method of Andrews (2005).

A small piece of tofu sample (<2 mm cube) was cut with a razor blade, and immediately immersed in liquid nitrogen for freeze fracture. This sample was subsequently freeze-dried overnight. After freeze drying, the sample was mounted on an aluminum stub with double sided carbon tabs. The surface of fractured tofu was positioned facing up. Then, the sample was earthed with conductive carbon paint.

The sample was put in the Polaron Sputter-Coated at 1.2 kV and 20 mA for 2 min. The sample was then placed under the microscope at 10 kV and 50 pA, and at 20 mm working distance. The scanning electron microscopy (SEM) images were captured at 2000x magnification. The examination was carried out in five replications from each treatment.

9.7 References

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APPENDICES

Appendix 1: The moisture content of soymilk, tofu and whey, and the density of tofu from all treatments of glutaraldehyde.

Treatment	Soymilk (Input)	Tofu (Output)		Whey (Output)
	Moisture content (%)	Moisture content (%)	Density (g/cm ³)	Moisture content (%)
Control	89.8 ± 0.2 (a,b)	76.0 ± 0.4 (a)	1.06 ± 0.02	96.7 ± 0.1 (a,b)
1 mM Glut B	88.4 ± 0.2 (a)	75.0 ± 0.3 (c)	1.04 ± 0.02	95.4 ± 0.2 (a,c)
2 mM Glut B	89.8 ± 0.7	75.4 ± 0.5	1.06 ± 0.01	97.4 ± 0.4 (c,d)
1 mM Glut A	88.6 ± 0.2 (b)	75.8 ± 0.2 (a,b,c)	1.04 ± 0.01	95.2 ± 0.2 (b)
2 mM Glut A	89.4 ± 0.4	77.6 ± 0.9 (b)	1.05 ± 0.01	96.2 ± 0.5 (d)
Control	89.8 ± 0.2	76.0 ± 0.4 (a,b,c)	1.06 ± 0.02	96.7 ± 0.1 (a,b,c,d)
15 mM Glut B (T5)	90.1 ± 0.3	76.0 ± 0.5 (d,e)	1.09 ± 0.02 (a)	97.1 ± 0.1 (a)
30 mM Glut B (T6)	90.1 ± 0.2 (b)	77.6 ± 0.3 (a,d,f)	1.05 ± 0.01	97.2 ± 0.1 (b)
15 mM Glut A (T7)	89.8 ± 0.3 (a)	79.1 ± 0.2 (b,e)	1.01 ± 0.01 (a)	97.0 ± 0.1 (c,e)
30 mM Glut A (T8)	89.4 ± 0.2 (a,b)	79.8 ± 0.7 (c,f)	1.06 ± 0.02	97.2 ± 0.1 (d,e)

Values are the means ± standard error of the means of five replicate measurements. Means in the same column followed by the same letter are significantly different ($P < 0.05$) among the treatments. Glut B = Added glutaraldehyde before boiling. Glut A = Added glutaraldehyde after boiling.

Appendix 2: The total volume of input and output in all treatments of glutaraldehyde.

Treatment	Total input	Output		Total output	Reduction in total output (%)	Total input versus Total output
	Soymilk (mL)	Tofu (mL)	Whey (mL)	Tofu + Whey (mL)		
Control	800 (ns)	290 ± 9 (a)	446 ± 11	736 ± 5 (a)	8	(**)
1 mM Glut B	800 (ns)	277 ± 2 (b)	438 ± 4	715 ± 3 (a,b)	11	(**)
2 mM Glut B	800 (ns)	264 ± 4 (a,c)	452 ± 4 (b)	716 ± 4	11	(**)
1 mM Glut A	800 (ns)	288 ± 2 (b)	443 ± 5 (a)	731 ± 4 (b)	9	(**)
2 mM Glut A	800 (ns)	293 ± 5 (c)	432 ± 4 (a,b)	725 ± 3	9	(**)
Control	800 (ns)	290 ± 9 (a,b)	446 ± 11 (a,b,c,d)	736 ± 5	8	(**)
15 mM Glut B	800 (ns)	292 ± 4 (c)	429 ± 10 (a,e)	721 ± 7 (a)	10	(**)
30 mM Glut B	800 (ns)	303 ± 12 (d)	427 ± 10 (b,f)	730 ± 4 (b)	9	(**)
15 mM Glut A	800 (ns)	365 ± 10 (a,c)	389 ± 6 (c,e)	754 ± 5 (a)	6	(**)
30 mM Glut A	800 (ns)	380 ± 14 (b,d)	366 ± 17 (d,f)	746 ± 5 (b)	7	(**)

Values are the means ± standard error of the means of five replicate measurements. Means in the same column followed by the same letter are significantly different ($P > 0.05$) among the treatments. ns = not significantly different ($P > 0.05$). (**) = significantly different at $P < 0.01$.

Glut B = Added glutaraldehyde before boiling; Glut A = Added glutaraldehyde after boiling.

Appendix 3: The total weight of input and output in all treatments of glutaraldehyde.

Treatment	Total input	Output		Total output	Reduction in total output (%)	Total Input versus Total output
	Soymilk (g)	Tofu (g)	Whey (g)	Tofu + Whey (g)		
Control	805 ± 1 (a,b,c,d)	276 ± 4 (a,b)	444 ± 10	720 ± 7	11	(**)
1 mM Glut B (T1)	812 ± 1 (a)	263 ± 2 (a,b,c,d)	443 ± 4	706 ± 3	13	(**)
2 mM Glut B (T2)	811 ± 1 (b)	247 ± 4 (c,e)	455 ± 5 (a)	702 ± 5	13	(**)
1 mM Glut A (T3)	810 ± 1 (c)	271 ± 2 (d)	447 ± 6	718 ± 6	11	(**)
2 mM Glut A (T3)	810 ± 1 (d)	273 ± 4 (e)	433 ± 4 (a)	706 ± 1	13	(**)
Control	805 ± 1	276 ± 4 (a,b,c)	444 ± 10 (a,b)	720 ± 7	11	(**)
15 mM Glut B (T5)	800 ± 4	272 ± 2 (d,e)	442 ± 12 (c)	714 ± 12	11	(**)
30 mM Glut B (T6)	799 ± 1 (a)	287 ± 4 (a,d,f)	434 ± 10	722 ± 7	10	(**)
15 mM Glut A (T7)	804 ± 2	318 ± 4 (b,e)	396 ± 6 (a,c)	714 ± 5	11	(**)
30 mM Glut A (T8)	804 ± 1 (a)	344 ± 12 (c,f)	372 ± 18 (b)	717 ± 6	11	(**)

Values are the means ± standard error of the means of five replicate measurements. Means in the same column followed by the same letter are significantly different (P<0.05). (**) = significantly different (P<0.01). Glut B = Added glutaraldehyde before boiling; Glut A = Added glutaraldehyde after boiling.

Appendix 4: The total water content of input and output in all treatments of glutaraldehyde.

Treatment	Total input	Output		Total output	Reduction in total output (%)	Total input versus Total output
	Soymilk (mL)	Tofu (mL)	Whey (mL)	Tofu + Whey (mL)		
Control	723 ± 3	209 ± 2 (a,b)	430 ± 10	639 ± 8	12	(**)
1 mM Glut B (T1)	718 ± 1	198 ± 1 (a,c,d)	424 ± 4	621 ± 3	13	(**)
2 mM Glut B (T2)	727 ± 5	187 ± 4 (b,c,e)	444 ± 5 (a)	630 ± 3	13	(**)
1 mM Glut A (T3)	718 ± 2	205 ± 1 (d)	426 ± 6	632 ± 5	12	(**)
2 mM Glut A (T4)	725 ± 4	212 ± 3 (e)	417 ± 6 (a)	629 ± 4	13	(**)
Control	723 ± 3	209 ± 2 (a,b,c,d)	430 ± 10 (a,b)	639 ± 8	12	(**)
15 mM Glut B (T5)	721 ± 6	206 ± 2 (e)	430 ± 11 (c)	636 ± 12	12	(**)
30 mM Glut B (T6)	720 ± 1	223 ± 2 (a,b,e,f)	422 ± 10	645 ± 8	11	(**)
15 mM Glut A (T7)	722 ± 4	251 ± 3 (c)	385 ± 6 (a,c)	636 ± 5	12	(**)
30 mM Glut A (T8)	718 ± 1	275 ± 12 (d,f)	362 ± 17 (b)	637 ± 6	11	(**)

Values are the means ± standard error of the means of five replicate measurements. Means in the same column followed by the same letter are significantly different ($P < 0.05$). (**) = significantly different ($P < 0.01$). Glut B = Added glutaraldehyde before boiling. Glut A = Added glutaraldehyde after boiling.

Appendix 5: The moisture content of soymilk, tofu and whey, and density of tofu in all treatments of TGA.

Treatment	Soymilk	Tofu		Whey
	Moisture content (%)	Moisture Content (%)	Density (g/cm ³)	Moisture Content (%)
Control	89.7 ± 0.1 (a)	75.8 ± 0.2 (a)	1.04 ± 0.01 (a)	96.9 ± 0.1 (a,b)
1000 ppm TGA-B	89.5 ± 0.1 (a)	75.8 ± 0.1 (b)	1.05 ± 0.02	96.8 ± 0.1 (a,c)
5000 ppm TGA-B	89.7 ± 0.1	75.4 ± 0.1 (b,d)	1.02 ± 0.01 (a)	96.9 ± 0.1 (b,d)
1000 ppm TGA-A	89.8 ± 0.2	76.5 ± 0.3 (c)	1.07 ± 0.01	97.1 ± 0.1 (c)
5000 ppm TGA-A	89.3 ± 0.4	77.7 ± 0.6 (a,c,d)	1.04 ± 0.02	97.0 ± 0.1 (d)

Values are the means ± standard error of the means of five replicate measurements. Means in the same column followed by the same letter are significantly different ($P < 0.05$). TGA-B = Added TGA before boiling; TGA-A = Added TGA after boiling.

Appendix 6: The total volume of input and output in all treatments of transglutaminase (TGA).

Treatment	Total input	Output		Total output	Reduction in total output (%)	Total input versus Total output
	Soymilk (mL)	Tofu (mL)	Whey (mL)	Tofu + Whey (mL)		
Control	800 (ns)	357 ± 2(a,b)	422 ± 2 (a,b)	779 ± 3 (a)	3	(*)
1000 ppm TGA-B	800 ns)	326 ± 6 (a,c)	423 ± 7	749 ± 6 (a,b)	6	(*)
5000 ppm TGA-B	800 (ns)	367 ± 3 (b,c)	413 ± 2 (a)	780 ± 2 (b)	3	(*)
1000 ppm TGA-A	800 (ns)	344 ± 18	411 ± 3 (b)	755 ± 15 (c)	6	(*)
5000 ppm TGA-A	800 (ns)	330 ± 19	417 ± 4	739 ± 14 (c)	7	(*)

Values are the means ± standard error of the means of five replicate measurements. Means in the same column followed by the same letter are significantly different ($P < 0.05$). ns = not significantly different ($P > 0.05$). * = significantly different ($P < 0.05$). TGA-B = Added TGA before boiling; TGA-A = Added TGA after boiling.

Appendix 7: The total weight of input and output in all treatments of transglutaminase (TGA).

Treatment	Total input	Output		Total output	Reduction in total output (%)	Total input versus Total output)
	Soymilk (g)	Tofu (g)	Whey (g)	Tofu + Whey (g)		
Control	812 ± 1 (a,b)	289 ± 1 (a,b,c)	427 ± 1	716 ± 2 (a,b,c)	12	(**)
1000 ppm TGA-B	799 ± 1 (a,c)	291 ± 2 (d,e)	424 ± 2	715 ± 3 (d,e)	11	(**)
5000 ppm TGA-B	799 ± 1 (b,d)	274 ± 2 (a,d,f)	424 ± 3	698 ± 2 (a,d,f)	13	(**)
1000 ppm TGA-A	813 ± 1 (c)	309 ± 6 (b,e)	424 ± 8	733 ± 3 (b,e)	10	(**)
5000 ppm TGA-A	817 ± 1 (d)	314 ± 2 (c,f)	420 ± 3	735 ± 3 (c,f)	10	(**)

Values are the means ± standard error of the means of five replicate measurements. Means in the same column followed by the same letter are significantly different ($P < 0.05$). ns = not significantly different ($P > 0.05$). (**) = significantly different ($P < 0.01$). TGA-B = Added TGA before boiling; TGA-A = Added TGA after boiling.

Appendix 8: The total water content of input and output in all treatments of transglutaminase (TGA).

Treatment	Total input	Output		Total output	Reduction in Total Output (%)	Total input versus Total output
	Soymilk (mL)	Tofu (mL)	Whey (mL)	Tofu + Whey (mL)		
Control	729 ± 1	217 ± 1 (a,b,c)	414 ± 1	632 ± 2 (a,b,c)	13	(**)
1000 ppm TGA-B	716 ± 1	220 ± 2 (d,e)	411 ± 2	631 ± 2 (d,e)	12	(**)
5000 ppm TGA-B	717 ± 1	207 ± 1 (a,d)	411 ± 3	618 ± 2 (a,d,f)	14	(**)
1000 ppm TGA-A	730 ± 1	237 ± 5 (b,e)	411 ± 8	648 ± 4 (b,e)	11	(**)
5000 ppm TGA-A	729 ± 3	244 ± 1 (c)	408 ± 3	652 ± 3 (c,f)	11	(**)

Values are the means ± standard error of the means of five replicate measurements. Means in the same column followed by the same letter are significantly different ($P < 0.05$). ns = not significantly different ($P > 0.05$). (**) = significantly different ($P < 0.01$). TGA-B = Added TGA before boiling; TGA-A = Added TGA after boiling.

